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Award Number: DAMD17-00-1-0522

TITLE: Mechanism of Transcriptional Regulation by Androgen Receptor and its Coactivators in the Context of Chromatin

PRINCIPAL INVESTIGATOR: Jiemin Wong, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

REPORT DATE: January 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2004	Final (1 Jul 2000 - 31 Dec 2003)

4. TITLE AND SUBTITLE

Mechanism of Transcriptional Regulation by Androgen Receptor and its Coactivators in the Context of Chromatin

5. FUNDING NUMBERS

DAMD17-00-1-0522

6. AUTHOR(S)

Jiemin Wong, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, TX 77030-3498

E-Mail: jwong@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Androgens play important roles in the differentiation, development and maintenance of male reproductive functions, as well as in the etiology of prostate cancer. The biological effects of androgens are believed to be mediated through the intracellular androgen receptor (AR), which belongs to the nuclear receptor (NR) superfamily of ligand-regulated transcription factors. Like other NRs, the actions of AR are subject to modulation, either positively or negatively, by an increasing number of co-regulatory proteins, termed coactivators or corepressors. Coactivators are believed to function either as bridging factors between receptors and basal transcription machinery to enhance recruitment of the basal transcription machinery and/or as factors that have capacity to actively remodel repressive chromatin. The purpose of this research is to study the mechanism by which coactivators modulate AR activity in chromatin, the physiological template of transcriptional regulation. In this progress report, we report that we have analyzed how SRC family coactivators, p300, ARA24 and ARA70 modulate AR activity in the context of chromatin using Xenopus oocyte as a model system. We show that expression of SRC-1 and p300 in Xenopus oocytes can significantly enhance both hormone-dependent and independent activation by AR. While ARA24 can stimulate modestly the AR activation, no coactivator activity can be observed for ARA70. We demonstrate that p300 requires both its histone acetyltransferase activity and interaction with SRC family coactivators to stimulate AR activity. By using chromatin immunoprecipitation assay, we demonstrated that R1881-stimulated transcriptional activation by AR is associated with the promoter targeting of multiple cofactors including the SRC-1, p300, SWI/SNF and TRAP/Mediator complex. An increased histone acetylation over the promoter region was also observed. This histone acetylation is correlated with the recruitment of CBP/p300. Taken together, our data suggest that hormone-dependent activation by AR is associated with two types of chromatin remodeling, histone acetylation and chromatin remodeling induced by SWI/SNF, as

14. SUBJECT TERMS

Prostate cancer, Androgen receptor, Coactivator, SRC1, p300, Chromatin

15. NUMBER OF PAGES

62

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Androgens play important roles in the differentiation, development and maintenance of male reproductive functions, as well as in the etiology of prostate cancer. The biological effects of androgens are believed to be mediated through the intracellular androgen receptor (AR), which belongs to the nuclear receptor (NR) superfamily of ligand-regulated transcription factors (1, 2). Like other NRs, AR is composed of distinct functional domains that include an amino-terminal domain that contains one or more trans-activation functions (AF1), a highly conserved DNA binding domain (DBD) and a multi-functional carboxyl-terminal ligand binding domain (LBD) that is involved in homo- or hetero-dimerization of the receptors, binding of specific ligands, and contains a ligand-dependent activation function (AF2) (1, 17).

Early studies indicate that in the absence of ligands, AR resides primarily in cytoplasm and is believed to associate with heat shock proteins in an inactive state (6, 15). Binding of ligand to AR is believed to trigger a series of events, including a change of conformation, translocation from the cytoplasm to the nucleus, and subsequent binding to specific promoter response elements, which eventually leads to activation or repression of its target genes (17). Recent studies indicate that the actions of AR are subject to modulation, either positively or negatively, by an increasing number of co-regulatory proteins, termed coactivators or corepressors (2, 10).

Coactivators are believed to function either as bridging factors between receptors and basal transcription machinery to enhance recruitment of the basal transcription machinery and/or as factors that have capacity to actively remodel repressive chromatin (2). Some coactivators such as ARA70 (20) or FHL2 (12) may be specific for AR, whereas many other coactivators, including SRC family coactivators, CBP, p300, PCAF and TRAP/DRIP/ARC complexes are generic to NRs [for review, see (2, 11)]. Importantly, many coactivators possess intrinsic histone acetyltransferase activity, whereas the corepressors such as SMRT and NCoR are found to associate with histone deacetylases in large protein complexes (3, 4, 9, 13). These findings provide a strong molecular connection between the modification of chromatin structure and transcriptional regulation by NRs.

Because packaging of eukaryotic DNA into chromatin has a general repressive effect on transcription, cofactors with chromatin remodeling activities are believed to have critical roles in gene expression. Two general classes of chromatin remodeling cofactors have been identified: histone-modifying enzymes and ATP-dependent chromatin remodeling factors (7). The significance of covalent histone modifications in transcriptional regulation is highlighted by the recent identification of large number of transcriptional cofactors as histone acetyltransferaseases (HAT), deacetylases, or methyltransferases. Acetylation of histone tails is believed to weaken the interaction of histones and DNA or affect the higher-order folding of nucleosomal arrays and thus enhances the access of nucleosomal DNA to sequence-specific transcription factors and Pol II basal machinery (14). In addition, histone modifications can also serve as code to modulate the interaction of specific proteins with chromatin (16).

The ATP-dependent chromatin remodeling factors, on the other hand, use the energy of ATP hydrolysis to alter nucleosome comformation and/or position, which in turn facilitate the access of DNA to transcription factors and Pol II basal machinery (7). Many different ATP-dependent

remodeling complexes have been identified including the SWI/SNF, the ISWI, and the Mi-2 families. The mammalian SWI/SNF are multi-subunit complexes of eight or more polypeptides in which the DNA-dependent ATPase is either the BRG1 or the BRM1 proteins. Recent studies indicate that the chromatin remodeling by SWI/SNF may involve histone octamer displacement, nucleosome sliding, and generation of negative supercoiling tension. Although both classes of chromatin remodeling factors are believed to facilitate transcription through their chromatin remodeling activities, the functional relationship between those two classes of chromatin remodeling factors is currently not clear. Recent studies in yeast indicate that SWI/SNF and GCN5, a histone acetyltrasferase, have both independent and overlapping functions in transcriptional activation (8).

In addition to chromatin remodeling factors aforementioned, another complex with a global effect on transcription is the Mediator complex (5). Both genetic and biochemical studies indicate that the Mediator, a protein complex with 20 subunit, is essential for basal and regulated expression of nearly all RNA polymerase II-dependent genes in the Saccharomyces cerevisiae genome. It is now clear that Mediator-like complexes, TRAP/SMCC, DRIP, ARC, also exist in higher eukaryotic cells and that they have an important and widespread role in metazoan transcriptional regulation. Mediator is believed to act as a bridge, conveying regulatory information from sequence-specific transcription factors that bind to the enhancers and/or the promoters to the Pol II basal transcription machinery.

The central question in gene expression is how the chromatin remodeling factors and the mediator complex are recruited to target genes by sequence specific transcription factors. The current prevailing view is that they are all recruited through direct interaction with various transcription factors. Indeed, both human and yeast SWI/SNF complexes, the GCN5/PCAF containing SAGA complex, CBP/p300, and the yeast and metazoan Mediator have been shown to interact directly with a number of transcription factors. Furthermore, in several cases such interactions have been shown to correlate with the activity of the transcription factors.

Our previous work and that of others have established *Xenopus* oocytes as an excellent model system for studies of transcriptional regulation by NRs in the context of chromatin (18, 19). *Xenopus* oocytes contain a large storage of factors required for transcription and both histones and non-histone proteins required for chromatin assembly. *Xenopus* oocytes are well suited for introduction of DNA, mRNA or proteins through microinjection. The purpose of this research is to study the molecular mechanisms by which AR regulates transcription in the context of chromatin. In our original proposal, we proposed to use Xenopus oocytes as a model system and to also establish a chromatin-based cell-free transcription system to study transcriptional regulation by AR and its coactivators in the context of chromatin. In this progress report, I summarize significant progress we have made so far on some of the tasks and also unexpected problems in others.

Body

The long-term objective of our original proposal is to understand the molecular mechanisms by which AR regulates transcription in prostate cancer. Since transcriptional regulation in eukaryotic cells takes place at the level of chromatin, we proposed to establish both an in vivo

and in vitro model systems to study how AR and its coactivators regulate transcription in the context of chromatin. Toward this goal, two specific aims were proposed previously:

Aim 1. To establish a Xenopus oocyte-based and a cell-free transcription systems to study the mechanisms by which AR and its coactivators function in the context of chromatin.

Aim 2. To study the transcriptional profile of the AR mutant (877Thr-Ala) frequently found in advanced human prostate cancer.

We have made significant achievement during the last three years of this funded proposal. However, as progress in science is often unpredictable, one can see from this summary report that we have exceeded some tasks we proposed three years ago but not the other. Next I will summarize the accomplishment for each proposed task.

Task 1. Generate the constructs for and express the expression of p300 and ARA70 in Xenopus oocytes

Task 2. Study the effect of coactivators SRC-1, p300, and ARA70 on AR activation in the context of chromatin in Xenopus oocytes.

We have accomplished both tasks (see attached manuscript I). Using Xenopus oocytes as a model system, we showed that SRC-1 and p300 enhance both ligand-dependent and –independent transcriptional activation by AR. In contrast, we find that expression of ARA70 in Xenopus oocytes had no effect on AR-stimulated transcription from either a MMTV-LTR driven reporter or a reporter bearing four consensus ARE and a minimal TK promoter (See Fig. 1), whereas another AR coactivator, ARA24, stimulated AR activation modestly in multiple experiments. Interestingly, a recent study showed that ARA70 is primarily a cytoplasmic protein, providing a potential explanation as to why we failed to observe a coactivator activity for ARA70 (21). In fact, we have managed to express the ARA70 to a various levels by injecting different concentration of ARA70 mRNA, yet we have not observed any significant effect on AR-stimulated transcriptional activation on many attempts (data not shown). We therefore conclude that, unlike SRC-1 and p300, ARA70 does not function as an AR-coactivator in our experimental system. Because much of ARA70 data published previously were all based on transient transfection assays, future work would be needed to examine whether ARA70 is a genuine coactivator for AR.

Task 3. Perform the structural and functional analysis of coactivators in Xenopus oocytes

We have focused our studies on coactivators p300 and SRC-1. We have generated a series of p300 mutants previously, including a mutant with severely reduced histone acetyltransferase activity. Transcriptional analysis in Xenopus oocytes indicated that, in comparison to the wild-type p300, this mutant was severely impaired in their ability to stimulate R-1881 stimulated activation by AR using MMTV reporter. This result indicates that remodeling chromatin through histone acetylation is an essential step for transcriptional activation from chromatin. This result was published recently in EMBO J (see attached manuscript II).

In collaboration with Dr. Bert W. O'Malley, we have also generated a series of SRC-1 mutants (data not shown). We have verified the expression of this set of mutants in *Xenopus* oocytes after

injection of their corresponding in vitro synthesized mRNA by Western blotting. We are currently determining the structural and functional domains of the SRC-1 important for AR activation using this set of mutants.

Task 4. Analyze the protein-protein interaction between coactivators

We have analyzed the protein-protein interaction between SRC family coactivators and p300. First, we confirmed by co-immunoprecipitation experiments the interaction between the SRC family coactivators and p300. Second, by using the p300ΔSRC mutant described above, we demonstrated that the interaction required the SRC interaction domain in p300. Recently, we have confirmed that the p300 interaction domain in SRC-1 is also important for the interaction. As shown in Fig. 2, expression of the SRC-1 interaction domain of the p300 can effectively inhibit the activation by liganded AR, thus indicating that the SRC-p300 interaction is functionally important for R1881-dependent activation. This result is consistent with the idea that CBP/p300 is recruited primarily through protein-protein interaction with SRC family coactivators.

Task 5. Purification of functionally active p300 protein from SF9 cells

We obtained p300 expression baculovirus from Dr. Lee Karus at Cornell University. By using a purification scheme as described, we affinity purified the His6-tagged p300 from baculovirus infected SF9 cells by using Ni-NTA agarose chromatography. As shown in Fig. 3, the purified p300 was at least 90% homologous based on the Coomassie blue staining after SDS-PAGE. Furthermore, such purified p300 proteins contained a potent HAT activity as revealed by HAT assay using core histones as substrate and was functionally active in enhancing T3-dependent activation by TR/RXR heterodimers in vitro (see attached manuscript IV).

Task 6. Preparation of functionally active ARA70 from injected Xenopus oocytes

Since we failed to observe any coactivator activity for ARA70 in our experiment system where SRC-1 and p300 can stimulate both hormone-dependent and –independent activation by AR, we conclude that ARA70 does not function directly as an AR coactivator. We therefore decided not to pursue this task.

Task 7. Establish a chromatin-based and AR-dependent R1881-responsive in vitro transcription system

Task 8. Study the effect of coactivators on AR activation in vitro in the context of chromatin

As shown in attached manuscript three, we showed recently that TR/RXR heterodimers are capable of stimulating transcription from several TRE-containing reporters assembled into chromatin in the presence of T3 in a cell-free transcription system. Furthermore, we showed that p300 and SRC family coactivators enhanced TR/RXR activation synergistically. The primary role of SRC proteins is to recruit CBP/p300, because the SRC derivative bearing the receptor interaction domain and CBP/p300 interaction domain is functionally equivalent to the full-length protein and because the p300 mutant with deletion of SRC interaction domain is severely impaired in its coactivator activity. Taken together, our work on TR/RXR demonstrates that a biochemically-defined, in vitro chromatin based transcription system is useful for addressing a number of questions related to the molecular mechanisms of transcription regulation.

Despite of our great effort, however, we have yet been able to establish an R1881-responsive, AR-dependent in vitro transcription system as we have done for TR/RXR. As in vitro transcription system and purified recombinant coactivators worked perfect well for TR/RXR, the major problem we have encountered so far is how to prepare functionally active recombinant AR proteins. So far we have prepared AR from insect cells and microinjected Xenopus oocytes. However, none of these preparations showed any hormone response in our in vitro transcription assay. Occasionally we observed a weak R1881-independent activation. It is not clear why purified recombinant AR is basically inactive in our in vitro trasncriptional system. A likely explanation is that purified recombinant AR proteins lost association with one or more chaperone proteins required for maintaining AR in a "hormone-responsive conformation". However, we also failed to observe any activation when less purified AR preparations were used. Our results are consistent with a recent report on analysis of AR activity using a cell-free transcription system. In this report, a weak AR-dependent, but R1881-independent transcription activity was observed.

Our failure to establish an AR-dependent, R1881-responsive in vitro transcription system represents a major challenge on AR research. So far, hormone-responsive in vitro transcription reactions have been established for all steroid/thyroid hormone receptors except AR and glucocorticoid receptor (GR). In fact, many laboratories have been trying extensively to establish an in vitro chromatin-based transcription system for AR, yet no one has successed so far. Despite of this setback, we will continue to work on this issue, since an in vitro transcription system is of significant importance in illustrating the molecular mechanisms by which AR regulates transcription.

Task 9. Assess the requirement for a specific coactivator in AR action by immunodepeletion

Due to the problem in establishing a R1881-dependent in vitro transcription system for AR, we have changed our strategy in this task. Instead we have been using various approaches to address this issue. We have recently shown that CBP/p300 is essential for R1881-dependent transcriptional activation by AR. Three lines of evidence support this conclusion. First, a p300 mutant with impaired HAT activity is defective in stimulating AR activation. Second, a CBP/p300 HAT specific inhibitor, Lys-CoA, can block the R1881-dependent transcriptional activation by AR. Third, expression of the SRC interaction domain derived from p300 can effectively block the activation by AR. Together these data indicate that CBP/p300 is essential for R1881-dependent activation by AR. For details of experiments, please see the attached manuscript IV.

Task 10-13. Study the transcriptional properties of the mutant AR (877Thr>Ala)

Regarding to the proposed work on the mutant AR (877Thr>Ala) found frequently in prostate cancer patients, we have generated this mutant AR by site-directed mutagenesis (data not shown). When tested in parallel, we found that this mutant AR can be expressed in Xenopus oocytes like the wild-type AR. As expected, this mutant AR is indistinguishable from the wild-type AR in response to R1881 and in the presence of coactivators like p300 and SRC-1 (data not shown). Unfortunately, we found that antagonists such as flutamide and casdex inhibited transcriptional activation by wild-type AR as well as by AR (877Thr>Ala) mutant, suggesting

that the differential response of AR(877Thr>Ala) mutant to AR antagonists may not be intrinsic to AR but cell-type dependent.

Other progress:
Although not proposed in original proposal, we have investigated the coactivators specifically recruited by AR during R1881-stimulated transcriptional activation by using chromatin immunoprecipitation (ChIP) assay. ChIP assay is a powerful method which allows to detect the proteins associated with DNA template (chromatin) during transcription. As shown in Fig. 4, we have analyzed by this method the association of SRC-1, p300, BRG1, TRAP220 and RNA polymerase II under the conditions with and without AR and with and without R1881. It is clear that in the presence of R1881 an increased association was detected for SRC-1, p300, BRG1, TRAP220 and RNA pol II. In agreement with the recruitment of p300, the levels of histone H3 and H4 acetylation were also increased. The finding that BRG1 is recruited by liganded AR also indicates the involvement of the ATP-dependent chromatin remodeling complex SWI/SNF in AR activation. The presence of TRAP220, a subunit of the TRAP/Mediator complex, is consistent with an essential, widespread role of this complex in transcription. Thus, the R1881-dependent transcriptional activation is correlated with the recruitment of multiple cofactors and histone acetylation. We are currently investigating how these distinct cofactors are recruited by liganded AR (direct or indirect) as well as the involvement of other AR cofactors such as ARA 70. The establishment of ChIP assay opens a new avenue for studying the molecular mechanisms by which AR regulates gene expression in the context of chromatin.

Statement of work accomplished/in progress

Task 1. Generate the constructs for and analyze the expression of p300 and ARA70 in Xenopus oocytes. **Accomplished.**

Task 2. Study the effect of coactivators SRC-1, p300, and ARA70 on AR activation in the context of chromatin in Xenopus oocytes. **Accomplished .**

Task 3. Perform the structural and functional analysis of coactivators in Xenopus oocytes. **Accomplished for p300 and SRC-1.**

Task 4. Analyze the protein-protein interaction between coactivators. **Accomplished for SRC-1 and p300.**

Task 5. Purification of functionally active p300 protein from SF9 cells. **Accomplished.**

Task 6. Preparation of functionally active ARA70 from injected Xenopus oocytes. **Not suitable.**

Task 7-8. Failed to establish but remain as a goal.

Task 9. Accomplished.

Task 10-12. Accomplished.

Key Research Accomplishments

- Expression constructs for SRC-1 and p300 have been generated.
- Expression of SRC-1 and p300 in Xenopus oocytes was verified.
- A R-1881 responsive AR-dependent transcription activation from Xenopus oocytes were established.
- A hormone-independent activation was observed when AR was expressed in high levels in both Xenopus oocytes and mammalian cells.
- A hormone-independent DNA binding activity of AR proteins expressed in Xenopus oocytes was demonstrated.
- A variety of mutants for p300 and SRC-1 were generated.
- Structural and functional analyses revealed that both SRC interaction and HAT activity were required for p300 to facilitate AR activation.
- Coactivators SRC-1 and p300 stimulate both hormone-dependent and independent activation by AR.
- Functionally active p300 proteins were prepared using a baculovirus expression system.
- Demonstrate that ATP-dependent chromatin remodeling factor SWI/SNF is required for AR activation.
- Demonstrate that the recruitment of SWI/SNF by AR is partially dependent on the histone acetylation.
- Demonstrate that the activation by AR is associated with the recruitment of SRC proteins, p300, SWI/SNF and the TRAP/Mediator complex by using chromatin immunoprecipitation (ChIP) assay.
- Demonstrate that the coactivator PRMT1 stimulates AR activation in a methyltransferase activity dependent manner.

Reportable Outcomes

The support from DOD has allowed us to make significant progress in our research. So far three manuscripts have been published (see attached manuscripts).

Conclusions

Significant progress has been made in most of the proposed tasks. We have established an AR-dependent, hormone-responsive transcription system using Xenopus oocytes. This has allowed us to demonstrate that coactivators SRC-1 and p300 stimulate AR activation in the context of chromatin. In so doing, p300 requires its interaction with SRC-1 and its intrinsic HAT activity. Furthermore, we observe that AR contains an intrinsic hormone-independent trans-activation activity. This activity is observed when a high level of expression of AR is achieved. This activity is observed in both Xenopus oocytes and mammalian cells. We believe this hormone-independent activity of AR may be relevant to the possible function of AR in hormone-independent prostate cancer (see attached manuscript I). In addition, we demonstrate that histone methylation is likely to play an important role in transcriptional regulation by AR, as the coactivator PRMT1 is found to be a histone H4 Arg-3 specific methyltransferase (see attached manuscript III). Finally, by using chromatin immunoprecipitation assay, we demonstrate that the activation by AR is associated with the recruitment of SRC proteins, p300, SWI/SNF and the Mediator complex. We show that transcriptional activation requires histone acetylation by

CBP/p300. We reveal a role of coactivator-coactivator and coactivator-histone interactions in recruitment multiple cofactors required for hormone-dependent activation by AR (see attached manuscript II). Taken together, we believe the establishment of a hormone-responsive chromatin-based transcription system using Xenopus oocytes will allow us to further elucidate the molecular mechanisms by which AR regulates transcription in the context of chromatin and by which AR retains its trans-activation function in hormone-independent prostate.

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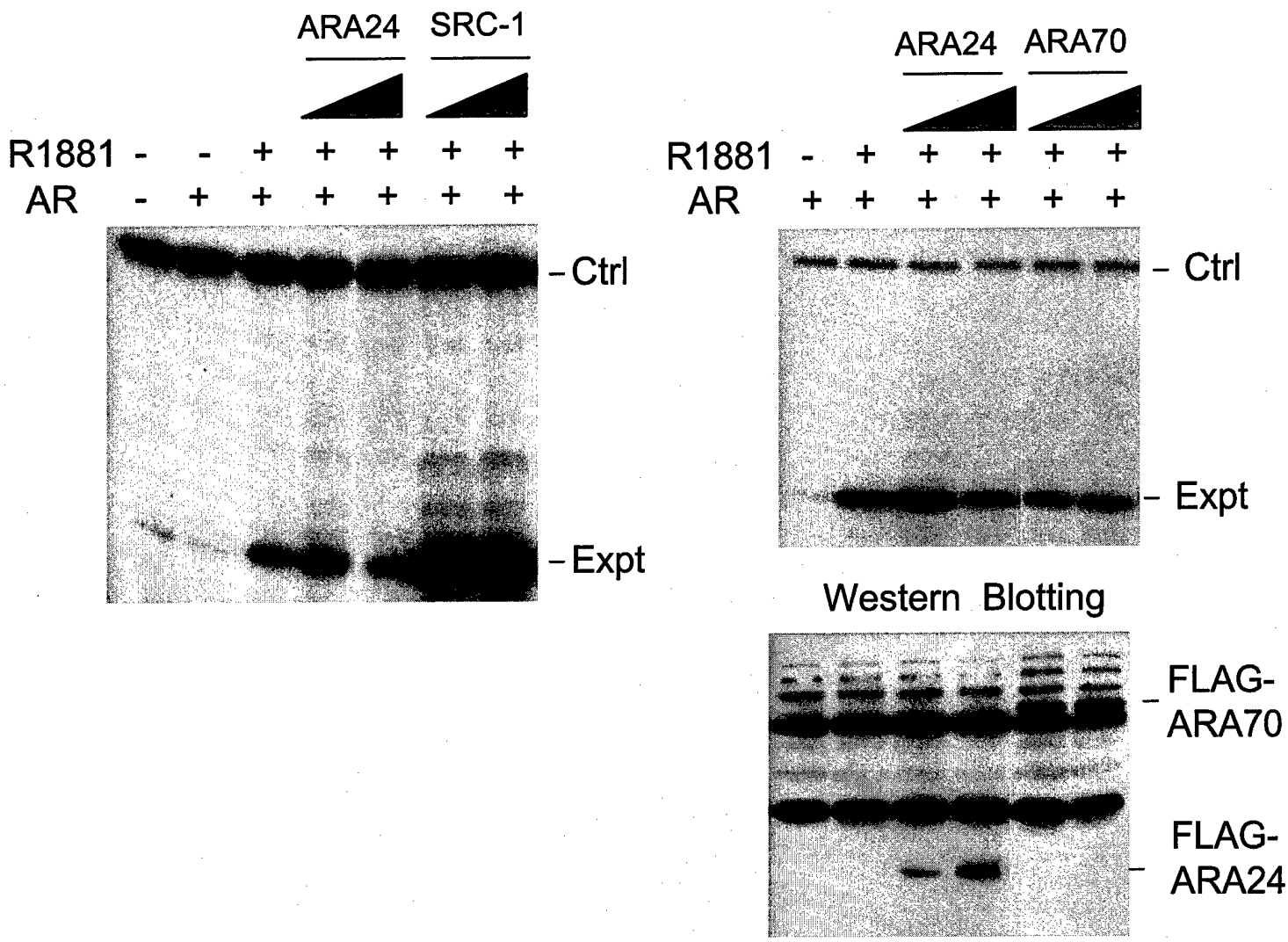

Fig. 1. Unlike SRC-1, ARA24 only modestly stimulated AR-dependent activation in Xenopus oocytes. The groups of oocytes were injected with a MMTV-LTR driven reporter, mRNAs encoding AR or various coactivators as indicated. A 3-fold difference in mRNA was used. After injection, oocytes were incubated with or without 50 nM of R1881 overnight and processed for analysis of transcription by primer extension. Ctrl, the primer extension product from the endogenous storage histone H4 mRNA. Expt, the primer extension product from MMTV-LTR driven transcription. (A) ARA24 is a weak coactivator in comparison to SRC-1. This conclusion is based on multiple experiments where various concentration of ARA24 were tested. (B) no coactivator activity was observed for ARA70. The lower panel shows both ARA24 and ARA70 were expressed.

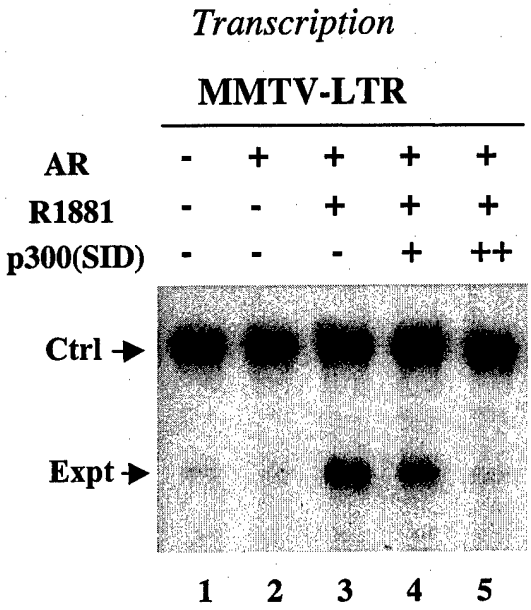

Fig. 2. The SRC-p300 interaction is essential for R1881-dependent AR activation. Groups of oocytes were injected with AR mRNA, MMTV-LTR reporter and a mRNA encoding the p300 SRC-interaction domain (SID, amino acids 2057-2170). Note that the activation was inhibited by p300(SID) in a dose dependent manner.

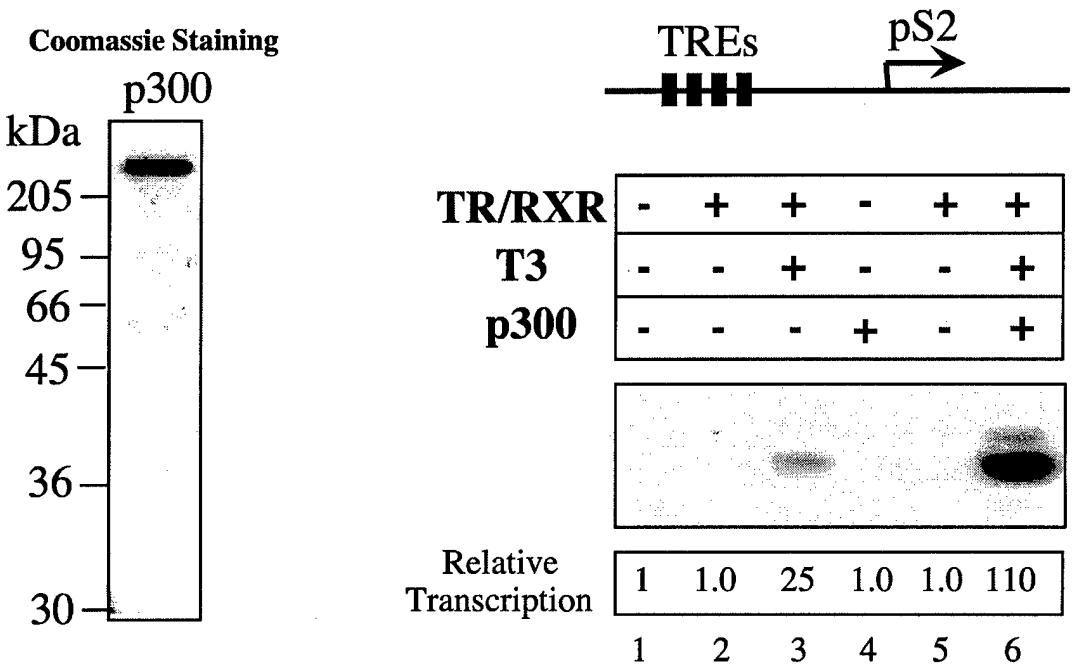

Figure 3. Purified recombinant p300 is functionally active in vitro. (A) Commassie blue staining of recombinant p300 purified from SF9 cells using Ni-NTA agarose beads. (B) The purified p300 stimulated T3-dependent activation by TR/RXR in a cell-free transcription system.

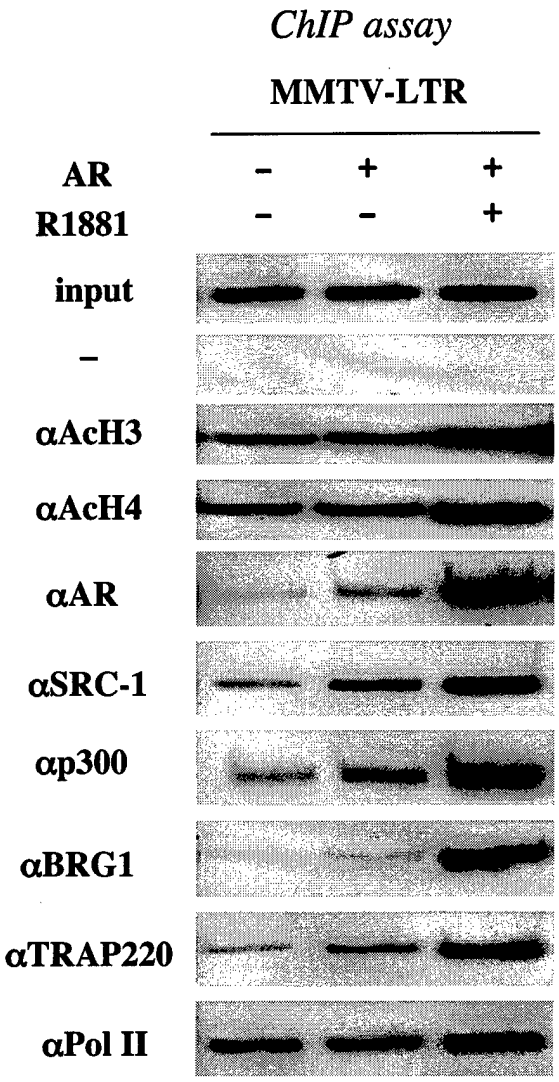

Fig. 4. Chromatin immunoprecipitation assay indicates the specific recruitment of multiple cofactors by liganded AR. The oocytes were injected with MMTV-LTR reporter and with or without AR as indicated. After incubation with or without R1881 overnight, the groups of oocytes were collected and processed for ChIP assay using specific antibodies as indicated. Note that the association of SRC-1, p300, BRG1, TRAP220 and Pol II were clearly increased in the presence of R1881 and AR, although Some increases in the absence of R1881were also observed. This increase most likely reflects the R1881-independent activation by AR.

Appendix

1. Wang H, Huang Z-Q, Xia L, Feng Q, Erdjument-Bromage H, Strahl BD, Briggs SD, Allis CD, Wong J, Tempst P, Zhang Y. 2001 Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. Science 293:853-857.

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Science Reprint

Methylation of Histone H4 at Arginine 3 Facilitating Transcriptional Activation by Nuclear Hormone Receptor

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3 August 2001, Volume 293, pp.853–857

particularly in lizards and birds, which commonly have derived nasal vestibules. The dearth of exceptions in the face of this diversity emphasizes the fundamental nature of the relationship.
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18. Why did anyone ever place the nostril caudally in dinosaurs? All leading early paleontologists regarded the largest dinosaurs (sauropods) as primarily "amphibious" (*32–35*). This view was clinched by the discovery in the sauropod *Diplodocus* of a large caudodorsal nasal opening (*36*) that was thought to serve as a snorkel, allowing the nearly submerged dinosaur to breathe (the dorsal opening in *Diplodocus* is actually just the caudal portion of the bony nostril, and the rostral portion extends far forward as a shallow narial fossa; vascular relationships confirm a rostral nostril). Although sauropods were later interpreted as terrestrial (*37*), the inferred caudal nostril stuck and somehow was transferred to other dinosaurs. Retracted nasal bones perhaps could be evidence, but nasal retraction is common in mammals in association with development of a proboscis, and mammals have been studied as extant analogs [e.g., (*1*, *38*)]. Despite retraction of the bony nostril, the fleshy nostril remains rostrally positioned; moreover, some mammals with retracted nasals (e.g., tapirs, elephants) enhance the fundamental amniote rostroventral position by extending the fleshy nostril out on a trunk. Thus, caudal expansion of the bony nostril apparently is not driven by caudal movement of the fleshy nostril, but rather by caudal expansion of the vestibular contents, that is, the narial apparatus.
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40. I thank S. W Cho and J. C. Sedlmayr for assistance with collecting data on the extant sample and S. D. Sampson for his role in the dinosaur studies. The manuscript was improved by comments provided by C. M. Holliday, J. S. McIntosh, P. M. O'Connor, S. D. Sampson, and J. C. Sedlmayr. S. M. Moody, K. de Queiroz, R. M. Elsey, and J. J. Baumel graciously provided some of the extant specimens. Funding was provided by NSF grants BSR-9112070 and IBN-9601174 and the Ohio University College of Osteopathic Medicine. The images on the cover and in Fig. 3 were skillfully painted by M. W. Skrepnick and W. L. Parsons, respectively, under the author's direction.

18 May 2001; accepted 27 June 2001

Methylation of Histone H4 at Arginine 3 Facilitating Transcriptional Activation by Nuclear Hormone Receptor

Hengbin Wang,[1] Zhi-Qing Huang,[2] Li Xia,[1] Qin Feng,[1] Hediye Erdjument-Bromage,[3] Brian D. Strahl,[4] Scott D. Briggs,[4] C. David Allis,[4] Jiemin Wong,[2] Paul Tempst,[3] Yi Zhang[1]*

Acetylation of core histone tails plays a fundamental role in transcription regulation. In addition to acetylation, other posttranslational modifications, such as phosphorylation and methylation, occur in core histone tails. Here, we report the purification, molecular identification, and functional characterization of a histone H4–specific methyltransferase PRMT1, a protein arginine methyltransferase. PRMT1 specifically methylates arginine 3 (Arg 3) of H4 in vitro and in vivo. Methylation of Arg 3 by PRMT1 facilitates subsequent acetylation of H4 tails by p300. However, acetylation of H4 inhibits its methylation by PRMT1. Most important, a mutation in the *S*-adenosyl-L-methionine–binding site of PRMT1 substantially crippled its nuclear receptor coactivator activity. Our finding reveals Arg 3 of H4 as a novel methylation site by PRMT1 and indicates that Arg 3 methylation plays an important role in transcriptional regulation.

Covalent modifications of core histone tails play important roles in chromatin function (*1*). One type of covalent histone modification is methylation (*2*), which has been observed in diverse organisms from yeast to human (*3*). However, the consequence of this posttranslational modification is not understood. One major obstacle in understanding the function of histone methylation is the lack of information about the responsible enzymes. The demonstrations that SUV39H1, the human homolog of the *Drosophila* heterochromatic protein Su(var)3-9, is an H3-specific methyltransferase (*4*) and that methylation of lysine 9 (Lys 9) on histone H3 serves as a binding site for the heterochromatin protein 1 (HP1) (*5–7*) underscore the importance of histone lysine methylation in heterochromatin function. Methylation of histones can occur on arginine residues, as well as lysine residues (*8*). The recent demonstrations that a nuclear receptor coactivator-associated protein, CARM1, is an H3-specific arginine methyltransferase suggests that histone arginine methylation may be involved in transcriptional activation (*9*).

To identify enzymes involved in core histone methylation, nuclear proteins from HeLa cells were separated into nuclear extract and nuclear pellet followed by further fractionation on DEAE52 and phosphate cellulose P11 columns. Fractions derived as above were assayed for methyltransferase activity by using core histone octamers as substrates (*10*). Multiple methyltransferase activities with distinctive specificity for histones H3 and H4 were seen (Fig. 1A). By following histone methyltransferase (HMT) activity (Fig. 1A), we purified an H4-specific HMT from the nuclear pellet fraction to homogeneity (*11*). Analysis of the column fractions derived from the hydroxyapatite column indicated that the peak of the enzymatic activity eluted in fraction 14 and trailed through fraction 26 (Fig. 1B, bottom panel). Silver staining of an SDS-polyacrylamide gel containing the column

[1]Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599–7295, USA. [2]Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. [3]Molecular Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10021, USA. [4]Department of Biochemistry and Molecular Genetics, University of Virginia Health Science Center, Charlottesville, VA 22908, USA.

*To whom correspondence should be addressed. E-mail: yi_zhang@med.unc.edu

fractions revealed that a polypeptide of 42 kD coeluted with the enzymatic activity (Fig. 1B, top panel). To confirm this result, the same input was loaded onto a gel-filtration Superose-200 column. Analysis of the column fractions indicated that the peak of the enzymatic activity eluted around 330 kD between fractions 38 and 41 (Fig. 1C, bottom panel). Silver staining of an SDS-polyacrylamide gel containing the column fractions revealed again that a 42-kD polypeptide coeluted with the enzymatic activity. Mass spectrometry analysis (*11*) identified the 42-kD polypeptide as the human protein arginine *N*-methyltransferase 1, PRMT1 (*12*). Because the HMT activity eluted around 330 kD and only coeluted with PRMT1, it is likely that PRMT1 functions as a homo-oligomer. This was verified by the demonstration that recombinant PRMT1 fractionated in the same way as the endogenous PRMT1, as a 330-kD complex (*11*). Therefore, we conclude that PRMT1 functions as an H4-specific HMT in the form of a homo-oligomer.

The identification of PRMT1 as one of the most abundant H4-specific HMTs is surprising, because only Lys 20 of H4 has been reported to be methylated in vivo (*1*) and because PRMT1 is not known to be able to methylate lysine residues. Instead, PRMT1 and its yeast homolog have been reported to mainly methylate arginine of certain RNA binding proteins (*8*). To determine whether PRMT1 methylates H4 on Lys 20, core histone octamers were methylated with recombinant or native PRMT1 in the presence of *S*-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H]SAM). After separation by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), methylated H4 was recovered and mi-

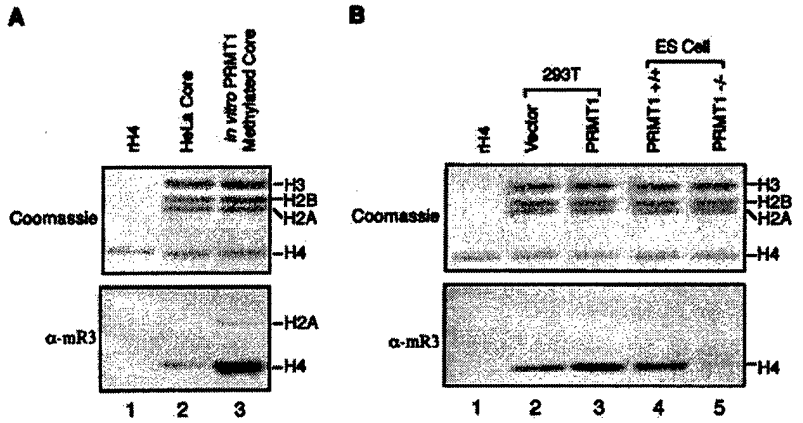

Fig. 2. PRMT1 methylates Arg 3 of H4 in vitro and in vivo. (**A**) Arg 3 methylation occurs in vivo. Recombinant histone H4 (200 ng) and equivalent amount of core histones from HeLa cells that were either subjected to mock (lane 2) or PRMT1 (lane 3) methylation before loading to SDS-polyacrylamide gel for Coomassie and Western blot analysis using the methyl-Arg 3–specific antibody. (**B**) PRMT1 is responsible for Arg 3 methylation in vivo. Recombinant histone H4 (200 ng) and equivalent amount of core histones purified from transiently transfected 293T cells (*23*) or ES cells were analyzed by Coomassie and Western blot as in (A).

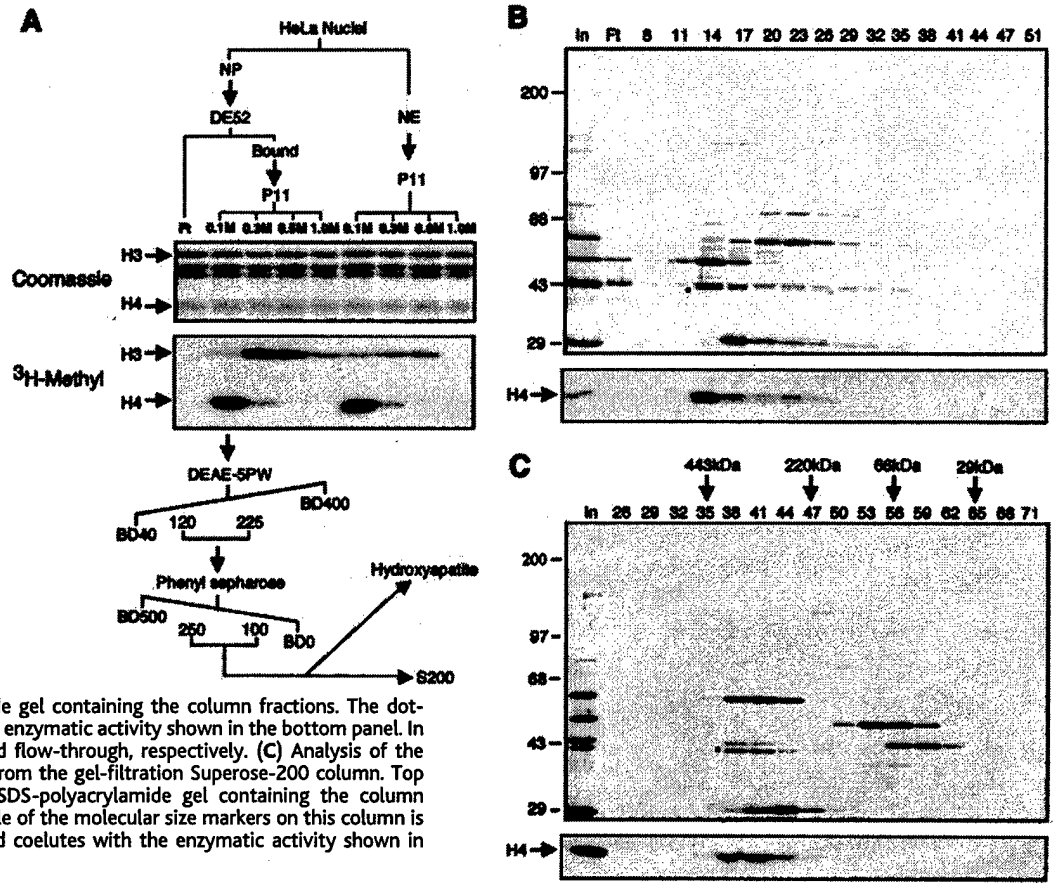

Fig. 1. Purification and molecular identification of an H4-specific methyltransferase. (**A**) HeLa nuclear proteins were fractionated as described (*11*) and fractions were assayed for histone methyltransferase activity (*10*). (Top) A Coomassie-stained gel indicating equal amounts of core histone substrate are used in each reaction. (Bottom) A fluorogram of the same gel indicating different fractions contain different HMT activities with specificity for H3 and/or H4. Equal volumes of the protein fractions were used in the enzymatic assay. Protein concentrations of the fractions from lanes 1 to 9 are 0.3, 0.3, 0.4, 0.4, 0.4, 1.2, 0.6, 0.3, and 1.2 mg/ml, respectively. (**B**) Analysis of the column fractions derived from the hydroxyapatite column. (Top) A silver-stained SDS-polyacrylamide gel containing the column fractions. The dotted-band coelutes with the enzymatic activity shown in the bottom panel. In and Ft represent input and flow-through, respectively. (**C**) Analysis of the column fractions derived from the gel-filtration Superose-200 column. Top panel is a silver-stained SDS-polyacrylamide gel containing the column fractions. The elution profile of the molecular size markers on this column is indicated. The dotted-band coelutes with the enzymatic activity shown in the bottom panel.

crosequenced by automated Edman chemical sequencing. Sequentially released amino acid derivatives were collected and counted by liquid scintillation, revealing that Arg 3, instead of Lys 20, was the major methylation site (*11*). Comparison of the ability of PRMT1 to methylate H4 tail peptides with or without a mutation on Lys 20 showed no difference, confirming that Lys 20 is not a site for PRMT1 methylation (*13*).

The identification of H4 Arg 3 as an in vitro methylation site for PRMT1 is intriguing. To determine whether Arg3 methylation occurs in vivo, antibodies against an Arg 3–methylated histone H4 NH_2-terminal peptide were generated (*14*). Although the antibody reacted strongly with PRMT1-methylated H4, it did not recognize equal amounts of recombinant H4 expressed in *Escherichia coli* (Fig. 2A, compare lanes 1 and 3), indicating that the antibody is methyl-Arg 3–specific. This same antibody also recognized histone H4 purified from HeLa cells (Fig. 2B, lane 2) indicating certain amount of Arg 3-methylation occurs in vivo. We note that H2A can also be weakly methylated by PRMT1 in vitro and that methylated H2A can be recognized by the methyl-Arg 3 antibody (Fig. 2A, compare lanes 2 and 3). The methylation site on H2A is likely to be Arg 3, because H2A has the same extreme NH_2-terminal sequence "SGRGK" as that of H4 (*14*). However, the amount of endogenous H2A methylation is undetectable under the same conditions (bottom panels of Fig. 2, A and B).

We next sought to determine whether PRMT1 is responsible for this site-specific Arg 3 methylation in vivo. If PRMT1 is responsible for Arg 3 methylation, overexpression of PRMT1 should increase the amount of Arg 3 methylation. The results shown in Fig. 2B indicate that overexpression of PRMT1 increases Arg 3 methylation (compare lanes 2 and 3). To confirm the above result, core histones from *PRMT1*$^{+/+}$ and *PRMT1*$^{-/-}$ embryonic stem (ES) cells (*15*) were purified and compared for their Arg 3 methylation. The results shown in Fig. 2B (compare lanes 4 and 5) demonstrated that inactivation of the *Prmt1* gene results in a dramatic decrease in the amount of Arg 3 methylation, indicating that histone H4 is likely an in vivo substrate for PRMT1. However, we could

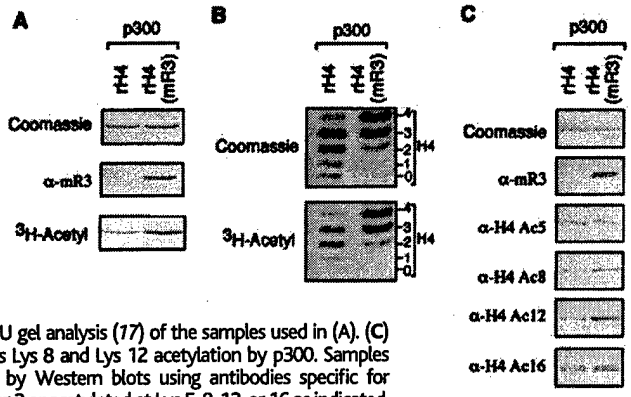

Fig. 3. Arg 3 methylation stimulates H4 acetylation by p300. (**A**) PRMT1-methylated H4 is a better substrate for p300 acetylation. Mock- and PRMT1-methylated recombinant H4 were subjected to p300 acetylation in the presence of [^{3}H]acetyl-CoA (*16*). Samples were analyzed by Coomassie, Western blot, and fluorogram. (**B**) TAU gel analysis (*17*) of the samples used in (A). (**C**) Arg 3 methylation facilitates Lys 8 and Lys 12 acetylation by p300. Samples used in (A) were analyzed by Western blots using antibodies specific for histone H4 methylated at Arg 3 or acetylated at Lys 5, 8, 12, or 16 as indicated. The site-specific acetyl-lysine antibodies are purchased from Serotec.

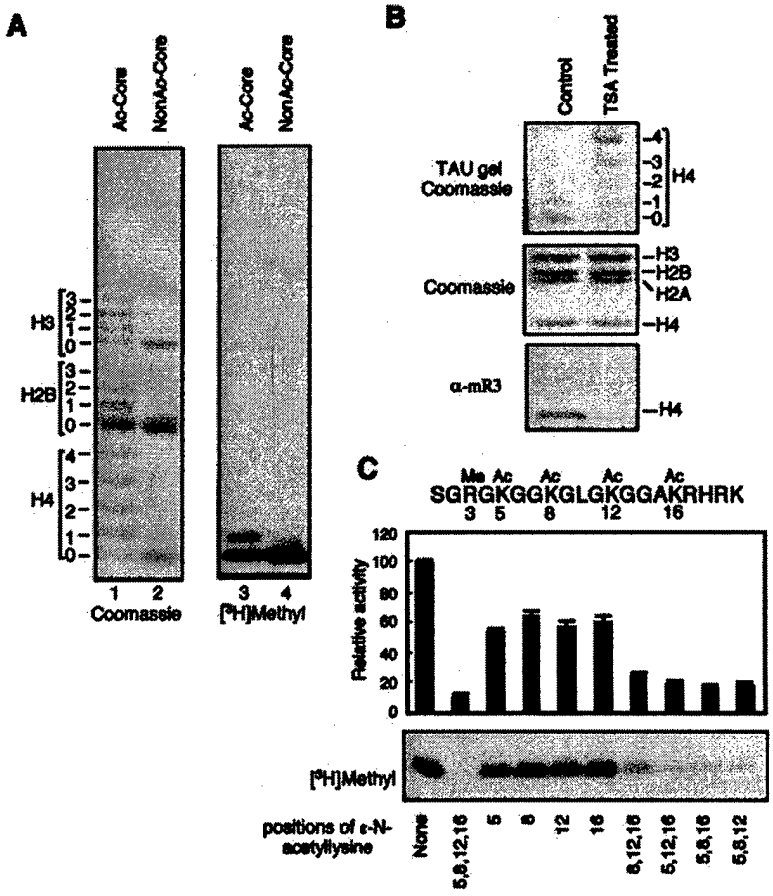

Fig. 4. Acetylation of H4 inhibits Arg 3 methylation by PRMT1. (**A**) Lysine acetylation inhibits H4-Arg 3 methylation in vitro. Hyper- (Ac) and hypoacetylated (Non-Ac) core histones purified from HeLa cells (*17*) were used as substrates for PRMT1 methylation (*10*). Different acetylated isoforms were resolved by a TAU gel and visualized by Coomassie staining. Methylation of different acetylated isoforms by PRMT1 was revealed by fluorogram. (**B**) Lysine acetylation inhibits H4-Arg 3 methylation in vivo. Core histones purified from untreated and TSA-treated (100 ng/ml final concentration) HeLa cells were analyzed by TAU gel (top panel), SDS-PAGE (middle panel), and Western blot (bottom panel). TAU gel reveals the acetylation state of H4, SDS-PAGE reveals equal loading, and Western blot reveals Arg 3 methylation state. (**C**) Comparison of the efficiency of PRMT1 to methylate different acetylated H4 peptides. Lysine residues that can be acetylated in vivo are indicated (top). Synthetic peptides that were not acetylated, monoed, triacetylated, and fully acetylated, respectively, were methylated with PRMT1 (*10*) and resolved by 20% SDS-PAGE before exposure to x-ray film (bottom). For quantification, the gel was cut and counted with scintillation counting. Results shown (middle) represent the average of two independent experiments with deviation.

not rule out the possibility that PRMT1 is an upstream regulator of an H4 Arg 3–specific HMT involved in H4 methylation through a methylation pathway similar to phosphorylation.

Having established that PRMT1 plays a critical role in Arg 3 methylation in vivo, we next sought to determine the function of this modification. Recent demonstration that methylation on Lys 9 of H3 inhibits Ser 10 phosphorylation (*4*) prompted us to ask whether Arg 3 methylation interferes with acetylation of lysine residues on H4 tails. To this end, we compared recombinant H4 that was either mock-methylated or PRMT1 methylated to serve as substrates for acetylation by p300 in the presence of [^{3}H]acetyl-CoA (*16*). Methylation of H4 by PRMT1 stimulated its subsequent acetylation by p300 (Fig. 3A). To confirm this result, samples equivalent to those analyzed in Fig. 3A were analyzed with a Triton-Acetic Acid-Urea (TAU) gel, which separates different acetylated histone isoforms. The results demonstrate that PRMT1-methylated H4 is a better substrate for p300 when compared with unmethylated H4, because all H4 molecules were acetylated (no 0 acetylated form) by p300 (Fig. 3B). However, under the same conditions, a fraction of the mock-methylated substrates still remains unacetylated (0 acetylated form). To determine which of the four acetylable lysine residues are affected by Arg 3 methylation, the acetylation status of samples analyzed above was examined by using acetylation site-specific antibodies. The results indicated that Arg 3 methylation facilitates K8 and K12 acetylation but has little affect on K5 or K16 acetylation (Fig. 3C).

To determine the effect of lysine acetylation on Arg 3 methylation, we purified both hyperacetylated and hypoacetylated core histones from HeLa cells (*17*) and used them as substrates for PRMT1 in the presence of [^{3}H]SAM. After methylation, samples were resolved in a TAU gel followed by Coomassie staining and autoradiography. Only un- and monoacetylated H4 isoforms were methylated to a detectable level, although nearly equal amounts of the different H4 isoforms were present in the methylation reaction (Fig. 4A, compare lanes 1 and 3). Because unacetylated H4 is the best substrate for PRMT1, when compared with different acetylated H4 isoforms (Fig. 4A), we concluded that acetylation on lysine residues inhibits H4 methylation by PRMT1. To determine whether this inhibition occurs in vivo, HeLa cells were treated with a histone deacetylase inhibitor, Tricostatin A (TSA), to induce hyperacetylation. Twelve hours after TSA treatment, core histones were isolated, and the methylation state of H4-Arg 3 was analyzed. Hypoacetylated H4 (untreated) had a higher amount of Arg 3 methylation when compared with hyperacetylated H4 (TSA treated), which had almost undetectable Arg 3 methylation (Fig. 4B). Therefore, hyperacetylation on lysine residues correlates with hypomethylation of H4 Arg 3. This result is consistent with the idea that acetylation on lysine residues inhibits its subsequent Arg 3 methylation, and it is also consistent with earlier studies demonstrating that H4 methylation preferentially occurs on unacetylated histones, whereas H3 methylation occurs preferentially on acetylated histones (*18*). Because H4 contains four lysine residues that can be acetylated (Fig. 4C, top panel), we investigated whether acetylation on any of the four sites would have a similar effect on Arg 3 methylation. To this end, synthetic H4 tail peptides that were not acetylated or were monoacetylated, triacetylated and fully acetylated, were used as substrates for PRMT1. Acetylation on any of the four lysines inhibited Arg 3 methylation by PRMT1 (Fig. 4C). However, acetylation on Lys 5 had the most effect. In addition, acetylation on different lysines seemed to have an additive inhibition effect. Consistent with results shown in Fig. 4A, triacetylated and fully acetylated peptides were severely impaired in serving as substrates for PRMT1 (Fig. 4C).

That Arg3 methylation enhanced lysine acetylation (Fig. 3) predicts that PRMT1 is likely to be involved in transcriptional activation. Indeed, PRMT1 has been shown recently to function as a coactivator of nuclear hormone receptors (*19*). However, its coactivator activity has not been linked to its HMT activity. To directly address the function of Arg 3 methylation on transcription, we introduced a single amino acid mutation (G80R) in the conserved SAM binding domain of PRMT1, which has been previously shown to impair its enzymatic activity (*20*). The ability of the mutant and wild-type PRMT1 to facilitate activation by androgen receptor (AR), which is known to use CBP/p300 as coactivators, was compared in chromatin context by using *Xenopus* oocytes as a model system (*21*). A mouse mammary tumor virus (MMTV) long terminal repeat (LTR)-based reporter was injected into the nuclei of *Xenopus* oocytes, and successful assembly of the reporter into chromatin was confirmed by micrococcal nuclease digestion (Fig. 5A). Ectopic expression of AR in *Xenopus* oocytes led to an agonist-stimulated activation of the reporter (Fig. 5B, compare lanes 2 and 3). Co-expression of PRMT1 further augmented the activation by AR (Fig. 5B, compare lanes 3 and 5). Significantly, the PRMT1(G80R) mutant has little coactivator activity when compared with wild-type PRMT1 (Fig. 5B, compare lanes 4 and 5 with lanes 6 and 7). Western blot analysis revealed that the differences in transcription were not due to differential expression of PRMT1 and PRMT1(G80R) or their effect on AR expression (Fig. 5B). We thus conclude that the HMT activity of PRMT1 is critical for its coactivator activity.

Our studies demonstrating the interplay between Arg3 methylation and lysine acetylation support the "histone code" hypothesis (*1*). We provided evidence that H4 Arg 3 methylation plays an important role in transcriptional activation. An H3-specific arginine methyltransferase CARM1 was also shown to function as a nuclear hormone receptor coactivator (*9*, *22*). In contrast, the heterochromatin-associated protein SUV39H1 was found to be an H3-specific methyltransferase (*4*), and methylation of Lys 9 by SUV39H1 serves as a binding site for the recruitment of the heterochromatin protein 1 (HP1) (*5*–*7*), suggesting that methylation of Lys 9 on H3 is likely involved in heterochro-

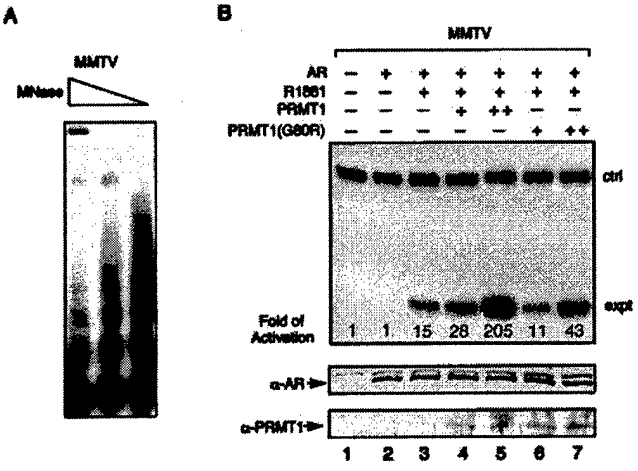

Fig. 5. The PRMT1 HMT activity is required for PRMT1 to function as a coactivator for AR. (**A**). The MMTV-LTR–based reporter injected into the nuclei of *Xenopus* oocytes was assembled into regularly spaced nucleosomes as revealed by Southern blot of a micrococcal nuclease digestion (MNase) assay (*11*). (**B**) Groups of *Xenopus* oocytes were injected with the MMTV-LTR reporter and the in vitro synthesized mRNAs encoding AR (100 ng/μl), PRMT1, or PRMT1(G80R) (100 ng/μl or 300 ng/μl) as indicated and were treated with or without the AR agonist R1881 (100 nM) overnight. The level of transcription from the reporter (expt) was analyzed by primer extension analysis of the total RNAs prepared from each group of oocytes and quantified by phosphor screen autoradiography (*11*). Folds of activation are shown below the primer extension product. The primer extension product from the endogenous histone H4 mRNA served as an internal control (ctrl). The expression levels of AR, PRMT1, and PRMT1(G80R) in each group of oocytes were analyzed by Western blot using an AR- or PRMT1-specific antibody, respectively.

matic gene silencing. Whether Arg 3 methylation helps the recruitment of specific histone acetyltransferases, such as p300, remains to be determined. As new HMTs responsible for the methylation of different histone arginine or lysine residues are identified, the functions of histone methylation on transcription and other processes involving chromatin will be revealed.

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10. Column fractions or recombinant PRMT1 was incubated with core histone octamers, recombinant H4, or H4 tail peptides in a total volume of 30 μl containing 20 mM Tris-HCl (pH 8.0), 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 1.5 μl [^{3}H]SAM (15 Ci/mmol; NEN Life Science Products) at 30°C for 1 hour. Reactions were stopped by the addition of SDS loading buffer followed by electrophoresis in an 18% SDS polyacrylamide gel. After Coomassie staining and destaining, gels were treated with Entensify (NEN Life Science Products) and dried before exposing to x-ray film.
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16. Recombinant H4 was purified as described (*24*) and used as substrates for PRMT1 methylation (*10*) in the presence of excess amounts of unlabeled SAM. Complete methylation was verified by the lack of further incorporation of [^{3}H]SAM. Acetylation was performed in a 20-μl volume containing 50 mM Hepes (pH 8.0), 5 mM DTT, 5 mM PMSF, 10 mM sodium butyrate, 10% glycerol, 2 μl [^{3}H]acetyl-CoA, and 2 μl of p300. Reaction mixture was incubated for 1 hour at 37°C and terminated by the addition of SDS sample buffer.
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16 March 2001; accepted 18 May 2001
Published online 31 May 2001;
10.1126/science.1060781
Include this information when citing this paper.

Identification of a Gene Associated with Bt Resistance in *Heliothis virescens*

Linda J. Gahan,[1] Fred Gould,[2] David G. Heckel[3]*

Transgenic crops producing insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely used for pest control. Bt-resistant insect strains have been studied, but the molecular basis of resistance has remained elusive. Here, we show that disruption of a cadherin-superfamily gene by retrotransposon-mediated insertion was linked to high levels of resistance to the Bt toxin Cry1Ac in the cotton pest *Heliothis virescens*. Monitoring the early phases of Bt resistance evolution in the field has been viewed as crucial but extremely difficult, especially when resistance is recessive. Our findings enable efficient DNA-based screening for resistant heterozygotes by directly detecting the recessive allele.

Field populations of the tobacco budworm *H. virescens*, a key pest of cotton and other crops in the Americas, have developed resistance to most classes of chemical insecticides. This species is the primary target of recently commercialized transgenic Bt cotton, which protects itself from insect damage by producing the insecticidal Cry1Ac toxin from *B. thuringiensis*. Concerns about Bt resistance led the U.S. Environmental Protection Agency to mandate a management plan, the "high-dose/refuge strategy" (*1*). It assumes that Bt cotton produces enough toxin to kill heterozygotes (with just one resistance allele) as well as

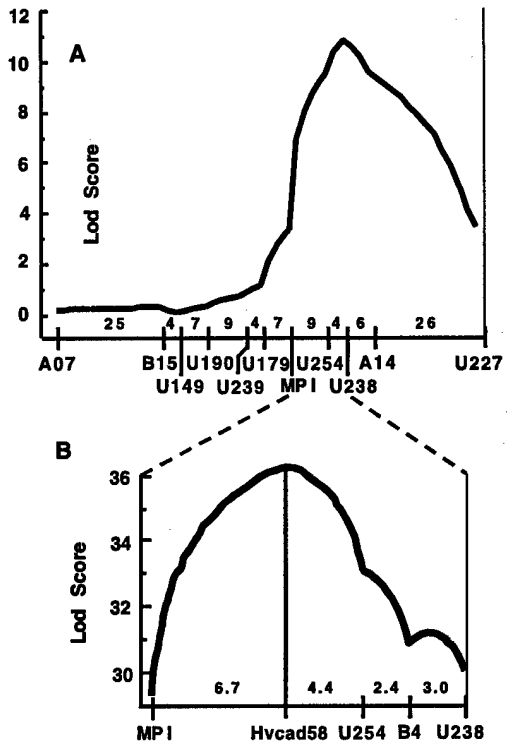

Fig. 1. QTL mapping of Cry1Ac resistance on linkage group 9 of *H. virescens*. (**A**) Resistance QTL lod (logarithm of the odds ratio for linkage) profile for initial scan of 105 cM on LG 9 spanned by 11 markers, based on 48 progeny of segregating backcross family D6. Marker order and spacing (in cM) was calculated by Mapmaker EXP 3.0 (*16*) and lod scores by Mapmaker QTL 1.9 (*17*). (**B**) Lod profile for fine-scale QTL mapping over the 16-cM region between MPI and U238, based on 268 progeny of nine segregating backcross families. The maximum lod score of 35.9 occurs at Hvcad58, which accounts for 46% of the trait variance. The resistance trait is the log of larval weight after 10 days of growth on 0.032 μg of Cry1Ac toxin per milliliter of diet (*3*).

0888-8809/02/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 16(5):924–937

AR Possesses an Intrinsic Hormone-Independent Transcriptional Activity

ZHI-QING HUANG, JIWEN LI, AND JIEMIN WONG

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030

Recent research has highlighted the functional importance of chromatin structure in transcriptional regulation. We have used *Xenopus* oocytes as a model system to investigate the action of AR in the context of chromatin. By manipulating the levels of AR expression, we have observed both agonist-dependent and -independent activation by AR. Expression of AR at relatively low levels resulted in strong agonist-dependent activation, whereas high levels of AR also led to hormone-independent activation. By using gel mobility shift and deoxyribonuclease I footprinting assays, we demonstrate that AR expressed in *Xenopus* oocytes binds to a consensus androgen response element *in vitro* in a ligand-independent manner. Expression of the coactivators steroid receptor coactivator-1, receptor-associated coactivator-3, and p300 stimulated both agonist-dependent and -independent activation by AR. Furthermore, this hormone-independent activity of AR is also observed in mammalian cells. Antagonists such as casodex can inhibit hormone-independent activity of AR, and this inhibition appears to correlate with the enhanced association with corepressor silencing mediator of retinoid and thyroid hormone receptors. Altogether, our studies reveal that AR has a capacity to activate transcription in a ligand-independent manner. (*Molecular Endocrinology* 16: 924–937, 2002)

ANDROGENS PLAY IMPORTANT roles in the differentiation, development, and maintenance of male reproductive functions, as well as in the etiology of prostate cancer. The biological effects of androgens are believed to be mediated through the intracellular AR, which belongs to the nuclear receptor (NR) superfamily of ligand-regulated transcription factors (1, 2). Like other NRs, AR is composed of distinct functional domains that include an amino-terminal domain that contains one or more *trans*-activation functions (AF1), a highly conserved DNA binding domain and a multifunctional carboxyl-terminal ligand binding domain that is involved in homo- or heterodimerization of the receptors, binding of specific ligands, and contains a ligand-dependent activation function (AF2) (1, 3–5).

Early studies indicate that in the absence of ligands, AR resides primarily in cytoplasm and is believed to associate with heat shock proteins in an inactive state (6, 7). Binding of ligand to AR is believed to trigger a series of events, including conformational change, translocation from the cytoplasm to the nucleus, and subsequent binding to specific promoter response elements, which eventually leads to activation or repression of its target genes (1, 4, 5). Like other NRs, research in the last several years has revealed an increasingly complexity of the mechanism of transcriptional regulation by AR (8). The actions of AR are subject to modulation, either positively or negatively, by an increasing number of coregulatory proteins, termed coactivators or corepressors (9). Coactivators are believed to function either as bridging factors between receptors and basal transcription machinery to enhance recruitment of the basal transcription machinery and/or as factors that have capacity to actively remodel repressive chromatin (8). While some coactivators such as ARA70 (10) or FHL2 (11) may be specific for AR, many of the coactivators identified so far, including steroid receptor coactivator (SRC) family coactivators, CREB-binding protein, p300, CBP/p300-associated factor, and TR-associated proteins/VDR-interacting proteins/activator-recruited cofactor complexes are generic to NRs (for review, see Refs. 8 and 9). Importantly, many coactivators possess intrinsic histone acetyltransferase activity (12). In contrast, corepressors such as silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (N-CoR) are found to associate with histone deacetylases in large protein complexes (13–16). These findings provide a strong molecular connection between the modification of chromatin structure and transcriptional regulation by NRs. Indeed, a conceptual advance in our understanding of transcription control over the last several years is the recognition of chromatin structure as an integral component of transcriptional regulation in eukaryotic cells (17). In comparison to other NRs such as TR, GR, PR,

Abbreviations: AF, Activation function; ARE, androgen response element; CAT, chloramphenicol acetyltransferase; DNase I, deoxyribonuclease I; ds, double-stranded; DTT, dithiothreitol; IP, immunoprecipitation; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; MNase, micrococcal nuclease; N-CoR, nuclear receptor corepressor; NR, nuclear receptor; RAC3, receptor-associated coactivator-3; SMRT, silencing mediator of retinoid and thyroid hormone receptors; SRC-1, steroid receptor coactivator-1; ss, single-stranded; TRβA, *Xenopus* thyroid hormone receptor βA; TRE, thyroid hormone response element.

and ER, little is known about how AR regulates transcription in the context of chromatin.

Uniquely among steroid hormone receptors, the hormone-dependent AF2 activity of AR is elusive. Deletion of the ligand binding domain generates an AR molecule with constitutive activity that in many transcription assays is equivalent to the activity of the full-length AR in the presence of ligands, whereas deletion of the N-terminal AF1 domain usually results in an AR molecule with low or no detectable activity even in the presence of ligands (6, 18). These observations suggest that AF1 contributes most, if not all, the activity of AR. Consistent with this idea, several studies indicate that the AF1 domain mediates primarily the interaction between the SRC family coactivators and liganded AR (19–21). Recent studies also indicate that a ligand-dependent intramolecular interaction between AF1 and AF2 domains is essential for AR transcriptional activity (22, 23). In addition, AR can be activated in the absence of androgens in different cell lines by growth factors such as IGF-I and epidermal growth factor or chemicals that directly activate the PKA signaling pathway (24, 25). The mechanism of such ligand-independent activation is not clear yet, but likely to involve phosphorylation of AR and/or its associated proteins.

Recent studies in prostate cancer provide evidence for the existence of a ligand-independent activity for AR. Androgens are known to play a crucial role in the occurrence and progression of prostate cancer. Patients with advanced prostate cancer are usually subjected to hormonal therapy by either androgen deprivation and/or blockade of AR with antiandrogens. These treatments are beneficial in the early stages of cancer but eventually lead to relapse of androgen-insensitive cancers (26). Paradoxically, many hormone-insensitive prostate cancers are found to be positive for both AR as well as the gene products that are regulated by AR (27–29), suggesting that AR may still remain functionally active and thus contribute to the progression of androgen-independent prostate cancer. While mutations in AR may lead to activation of AR in the absence of ligands or a change in its hormone specificity, recent studies indicate that mutations in AR are rare events in hormone-insensitive cancers. Instead, the amplification and consequent overexpression of the wild-type AR gene appears to be the most common event found in hormone-refractory prostate cancer (29). These observations have led to the hypothesis that overexpression of AR and its subsequent activation by growth factor-mediated cross-talk pathways could lead to the ligand-independent activation of AR in hormone-insensitive prostate cancer. However, it is not known whether overexpression of AR alone is able to activate transcription in the absence of cross-talk pathways.

An important question related to the issue of the hormone-independent activity is whether AR can bind to an androgen response element (ARE) in the absence of ligand. Although ligand is usually required for androgen-dependent transcription activation because AR is located primarily in cytoplasm in the absence of ligand, the fact that AR can be activated by other signaling pathways in the absence of ligand argues that AR has the capacity to bind DNA in a ligand-independent manner. So far, *in vitro* gel shift assays have yielded conflicting results on this subject. In some cases, *in vitro* translated AR or AR produced in insect cells is capable of binding to AREs *in vitro* in the absence of ligand (30–32), whereas in other cases pretreatment with ligand is required for DNA binding *in vitro* (33). The discrepancy over whether AR can bind DNA in the absence of ligands *in vitro* is at least partly due to the technical difficulty in producing sufficient amounts of recombinant unliganded AR proteins and further complicated by the fact that AR appears to have an intrinsic weak DNA binding activity.

Our previous work and that of others have established *Xenopus* oocytes as an excellent model system for studies of transcriptional regulation by NRs in the context of chromatin (34, 35). *Xenopus* oocytes contain a large storage of factors required for transcription and both histones and nonhistone proteins required for chromatin assembly. *Xenopus* oocytes are well suited for introduction of DNA, mRNA, or proteins through microinjection. Introduction of DNA into the nucleus of *Xenopus* oocytes through microinjection allows the assembly of injected DNA into chromatin through two different pathways depending upon the type of DNA injected. While microinjection of DNA templates either as single-stranded (ss) or double-stranded (ds) DNA into *Xenopus* oocyte nucleus leads to the assembly of both DNA templates into chromatin, the chromatin template resulted from injection of ssDNA is more refractory to basal transcription than that generated by dsDNA. This is because that the ssDNA injected into *Xenopus* oocyte nucleus is rapidly converted into dsDNA through the synthesis of the complementary strand. The resulting dsDNA is assembled into chromatin within 30 min after injection in a process coupled to the synthesis of the complementary strand (replication-coupled assembly pathway) (34, 36), which mimics the chromatin assembly process during S phase in cell cycle.

In this study, we have reconstituted a ligand-responsive AR transcriptional system using *Xenopus* oocytes in an effort to understand the molecular mechanisms of transcriptional regulation by AR in the context of chromatin. We demonstrate that, while R1881 strongly stimulated transcriptional activation by AR, a ligand-independent activity is also observed when AR is highly expressed. Expression of coactivators such as members of SRC family and p300 stimulates both ligand-independent and -dependent activation by AR. *In vitro* DNA binding assays indicate that ligand is not required for AR DNA binding activity. Furthermore, this hormone-independent activity is also observed in mammalian cells. Interestingly, addition of AR-antagonists such as casodex can inhibit this hormone-independent activity and this inhibitory effect appears to correlate with

the recruitment of corepressor SMRT. Taken together, our results indicate that overexpression of AR can lead to activation of AR target genes in a ligand-independent manner and thus provide a possible molecular basis for the roles of AR gene amplification and consequent overexpression of AR in many hormone-refractory prostate cancers.

RESULTS

Xenopus Oocytes as a Model System for AR

To gain insight into how AR regulates transcription in the context of chromatin, we chose to use the *Xenopus* oocyte as a model system. To express AR in *Xenopus* oocytes, oocytes were injected with *in vitro* synthesized mRNA encoding a FLAG-tagged human AR and incubated overnight. Subsequent Western analysis using an AR-specific antibody (N-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) revealed the presence of AR in the extract derived from the oocytes injected with AR mRNA (Fig. 1A, lane 2), whereas no endogenous AR proteins could be detected from the noninjected control oocyte extract (Fig. 1A, lane 1). Indeed, Western analysis using a different AR antibody (C-19; Santa Cruz Biotechnology, Inc.) also failed to detect the presence of AR protein in *Xenopus* oocytes (data not shown), indicating that *Xenopus* oocytes contain a very low level, if any, of endogenous AR proteins.

To investigate transcriptional regulation by AR in chromatin, we used two reporter constructs. Our previous work demonstrated that chromatin structure is important for transcriptional regulation of the *Xenopus* TRβA promoter by TR (34). We thus generated a TRβA promoter-based reporter (4·ARE-TRβA) by inserting four copies of a consensus ARE upstream of the TRβA transcriptional start site (Fig. 1B). Because the functional importance of chromatin structure in transcriptional regulation of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) by steroid hormone receptors has been well established (37), we also generated a MMTV-LTR-based reporter (Fig. 1B). To assemble reporter DNA into repressive chromatin through the replication-coupled chromatin assembly pathway (36), we injected both reporters in ssDNA form into the nucleus of *Xenopus* oocytes. After overnight incubation, the injected oocytes were collected and the chromatin structure was analyzed by micrococcal nuclease (MNase) digestion assay. As shown in Fig. 1C, limited MNase digestions revealed that injection of both reporters as ssDNA plasmids led to the assembly of the DNA into chromatin with regularly spaced nucleosomes. This result is consistent with the notion that injection of ssDNA plasmid will result in efficient assembly of chromatin through a replication-coupled assembly pathway.

We next examined whether expression of AR could activate transcription from repressive chromatin in *Xenopus* oocytes. Groups of *Xenopus* oocytes were injected with mRNA encoding AR (100 ng/μl, 18.4 nl/oocyte) and ssDNA of the MMTV reporter and treated with agonist R1881 or the antagonists casodex or flutamide at concentrations as indicated (Fig. 2). After overnight incubation, the total RNA was purified from each group of oocytes and the level of transcription from the MMTV promoter was analyzed by primer

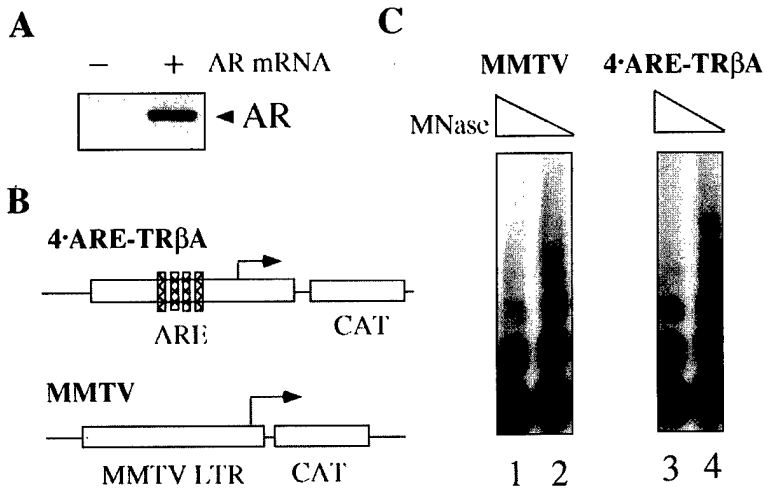

Fig. 1. Expression of AR and Assembly of AR-Responsive Reporters into Chromatin in *Xenopus* Oocytes through Microinjection

A, Western analysis using an AR-specific antibody (N-20, Santa Cruz Biotechnology, Inc.) of extracts derived from control oocytes (–) and oocytes injected with AR mRNA (+) (100 ng/μl, 18.4 nl/oocyte). B, Diagram showing the structure of 4·ARE-TRβA promoter-based and MMTV LTR-based reporters. The *arrow* indicates the transcriptional start site. C, Both reporters were assembled into chromatin with regularly spaced nucleosomal arrays via replication-coupled pathway. The ssDNA of both reporters was injected into the nucleus of *Xenopus* oocytes (50 ng/μl, 18.4 nl/oocyte). After overnight incubation, the chromatin structure was analyzed by MNase assay as described in *Materials and Methods*.

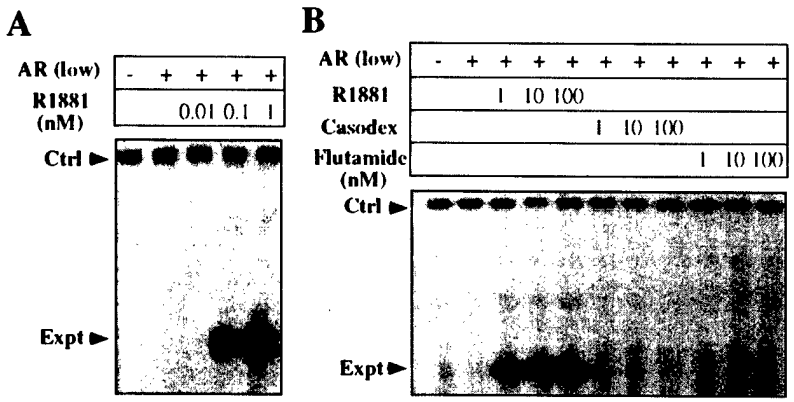

Fig. 2. R1881 But Not the Antagonists Casodex and Flutamide Stimulates AR Transcriptional Activation

Groups of oocytes were injected with a low dose of AR mRNA (100 ng/μl, 18.4 nl/oocyte) and ssDNA of MMTV reporter (50 ng/μl, 18.4 nl/oocyte). The oocytes were then treated overnight with R1881 or the antagonists casodex or flutamide at a concentration as indicated in (A) and (B). The levels of transcription were then analyzed by primer extension assay. Ctrl, The primer extension product derived from the endogenous storage histone H4 mRNA. Expt, The primer extension product derived from transcripts from the MMTV LTR reporter using end-labeled CAT primer as described in *Materials and Methods*.

extension assay. A histone H4-specific primer, which detected the endogenous histone H4 mRNA and thus served as an internal loading control, was included in the primer extension reaction. As shown in Fig. 2, addition of R1881 at concentrations of 0.1 nM was sufficient to activate transcription from the MMTV promoter, whereas addition of casodex or flutamide in a concentration ranging from 1 nM to 100 nM failed to do so. Similar results were observed when the 4.ARE-TRβA reporter was used (data not shown). We thus conclude that AR expressed in *Xenopus* oocytes exhibits the expected hormone specificity and activates transcription from the MMTV LTR assembled into chromatin.

We next tested the effect of the levels of AR protein on transcriptional activation from both MMTV and TRβA-based reporters. Groups of oocytes were injected with a low dose (100 ng/μl) or a high dose (1 μg/μl) of AR mRNA and the reporter DNA (ssDNA) as indicated (Fig. 3A). Levels of transcription were assayed after overnight incubation in the presence or absence of R1881 (10 nM). Consistent with the result in Fig. 2, a R1881-dependent activation was observed from both the MMTV- and TRβA-based reporters when a low concentration of AR mRNA (100 ng/μl) was injected (Fig. 3A). However, an R1881-independent activation of transcription from both reporters (compare lanes 4 with 2 and lanes 9 with 7) was clearly detected when a high dose of AR mRNA was injected. Although addition of R1881 led to a stronger final levels of transcription (compare lanes 5 with 3 and lanes 10 with 8), the fold of R1881-dependent activation actually decreased due to the presence of R1881-independent activation. On the TRβA promoter, both R1881-independent and -dependent activation required the presence of AREs, because no activation was observed when the parental reporter without AREs was used as the reporter (compare lanes 12 and 13 with 11), indicating that both R1881-dependent and -independent activation were directly mediated by AR. Because ligand-independent activation for AR has only been reported in the cases of activation by cross-talk pathways and because the ligand-independent activity of AR has been implicated clinically in hormone-refractory prostate cancer, we focus here on the characterization of the molecular mechanism of this R1881-independent transcriptional activation by AR.

Increased AR Expression Leads to Increased Nuclear Distribution of AR

Because studies in mammalian cells demonstrated that in the absence of ligand AR resides primarily in cytoplasm (6, 7), we first examined whether AR expressed in the *Xenopus* oocytes also exhibited a similar distribution. To do this, we took the advantage of the fact that the nucleus of *Xenopus* oocytes can be easily dissected manually away from the cytoplasm. Groups of *Xenopus* oocytes were injected with the low and high dose of AR mRNA as in Fig. 3A and incubated with or without addition of 10 nM of R1881. After overnight incubation, nuclear, cytoplasmic and total oocyte fractions were prepared. Due to the drastic difference in volume between the nucleus and cytoplasm of *Xenopus* oocytes (38), total proteins equivalent to three nuclei, half an oocyte of cytoplasmic and half an oocyte of the total oocyte extracts were fractionated by using a SDS-PAGE, and the distribution of AR was analyzed by Western blotting. As shown in Fig. 3B, in the absence of R1881, the majority of AR was found in the cytoplasm in both groups of oocytes injected with the low and high doses of AR mRNA (compare lanes 1 and 2). Treatment with R1881 led to a strong enrichment of AR in the nuclear fraction (compare lanes 5 and 4). This result indicates that AR expressed in *Xenopus* oocytes is primarily localized to the cytoplasm in the absence of R1881 and undergoes translocation to the nucleus in response to R1881

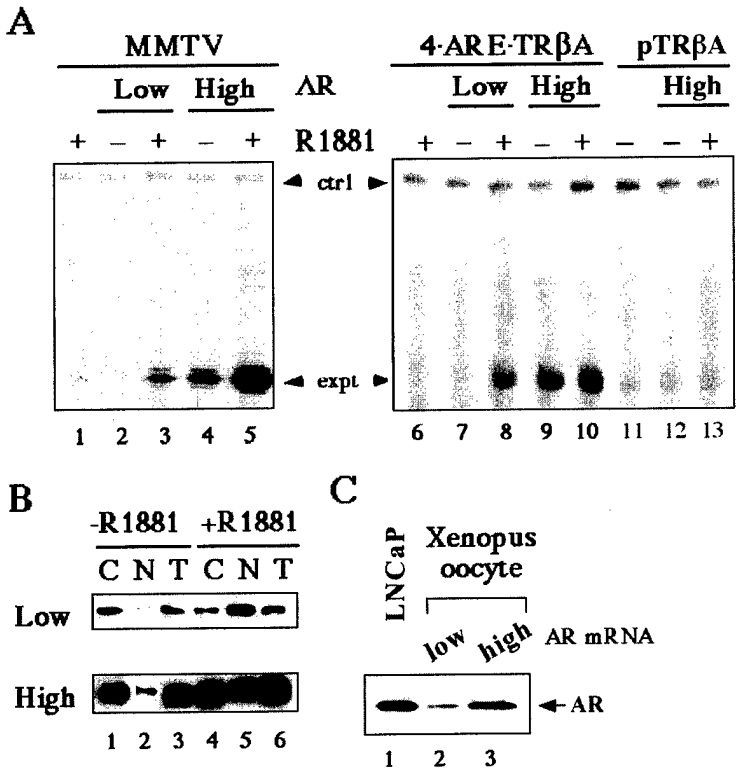

Fig. 3. AR Exhibits Both R1881-Dependent and -Independent Activation

A, Injection of low and high doses of AR mRNA led to observation of both R1881-dependent and -independent activation. Groups of oocytes were injected with AR mRNA at low (100 ng/μl, 18.4 nl/oocyte) or high concentrations (1 μg/μl, 18.4 nl/oocyte) and treated with or without R1881 (10 nM) overnight. All reporters were injected as ssDNA as in Fig. 1C. The primer extension assay was as in Fig. 2B for MMTV reporter and the primer I for pTRβA-based reporters. Note that AR failed to activate transcription from the control pTRβA reporter (without AREs). B, Subcellular localization of AR expressed in *Xenopus* oocytes. Groups of oocytes were injected with AR mRNA at low (100 ng/μl, 18.4 nl/oocyte) or high concentrations (1 μg/μl, 18.4 nl/oocyte) and treated with or without R1881 (10 nM) overnight as in A). The nuclear (N) and cytoplasmic (C) fractions of the oocytes were then dissected manually and analyzed for AR proteins by Western blotting using a FLAG-tag-specific antibody (M2, Sigma). T, Total oocyte extract. Note that protein extracts equivalent to 3 nucleus, half an oocyte of cytoplasm, and half a total oocyte were used here for Western analysis. C, Comparison of the levels of AR expressed in *Xenopus* oocytes with that in LNCaP cells. Total cell extract from LNCaP cells (5 μg) and total extracts (5 μg) prepared from oocytes injected with low and high doses of AR mRNA as in panel B were compared by Western analysis using an AR-specific antibody.

treatment. Thus, the pattern of subcellular localization of AR proteins in *Xenopus* oocytes is identical with that in mammalian cells.

Importantly, as shown in Fig. 3B, injection of the high dose of AR mRNA (1 μg/μl) clearly increased the level of AR protein in the nucleus in the absence of R1881 (compare lane 2 in the low and high). This result, together with the requirement of AREs for both R1881-dependent and -independent activation, suggests a model in which overexpression of AR leads to an increased level of AR protein in the nucleus, and this nuclear AR leads to subsequent activation of transcription even in the absence of R1881.

As the hormone-independent activation was only clearly observed when a high dose of AR mRNA was injected, we were concerned whether this is a phenomenon that exists only in the presence of vastly overexpression of AR. To have some sense about the levels of AR proteins, we compared the levels of AR proteins in *Xenopus* oocytes primed with low and high doses of AR mRNA with that that in an AR-positive prostate cancer cell line, LNCaP. When the same amount of the total proteins (5 μg) of LNCaP whole cell extract or AR mRNA primed oocyte extracts were analyzed for levels of AR by Western blotting (Fig. 3C), we found that level of AR in LNCaP cells was even higher than that in oocyte extract primed with the high dose of AR mRNA. This result indicates that a comparable level (concentration) of AR proteins can be found in prostate cancer cells such as LNCaP cells and thus suggests that the hormone-independent transcriptional activation by AR may have clinical relevance.

DNA Binding *in Vitro* by AR Protein Is Ligand Independent

The capacity of AR to activate transcription in the absence of hormone implies that AR can bind DNA in the absence of ligand. Because it is controversial as to whether ligand is required for DNA binding by AR, we

analyzed the DNA binding activity of AR proteins expressed in *Xenopus* oocytes. We first carried out gel mobility shift assays using a ^{32}P-labeled ARE-containing oligonucleotide probe and oocyte extracts prepared from oocytes injected with AR mRNA and treated with or without R1881 (10 nM). To maintain the association with R1881 of the AR derived from the R1881-treated AR-expressing oocytes, a final concentration of 10 nM of R1881 was added to all buffers used for binding assay or for making extracts derived from the R1881-treated oocytes. As shown in Fig. 4, a shifted DNA complex can be observed in lanes with both AR programmed extracts, with (lane 3) or without R1881 (lanes 8), but not in the lanes with control oocyte extract (lanes 2 and 7). In addition, this complex is ARE specific because the complex could be eliminated by addition of an excessive cold ARE competitor but not cold TRE competitor. Furthermore, in multiple experiments, we observed that the AR-DNA complex in the presence of R1881 appeared to migrate slightly slower than that in the absence of R1881 [compare lane 3 with lane 8 and use the nonspecific complex indicated by an *asterisk* (*) as a reference] suggest this difference in mobility may reflect the conformational changes of AR or/and the AR-DNA complex after binding of R1881.

Next, we carried out deoxyribonuclease I (DNase I) footprinting assays to ensure that AR indeed bound to the ARE in a sequence-specific manner. For this purpose, a ^{32}P-labeled DNA fragment from the TRβA promoter containing a single ARE insertion was generated by PCR and used as probe. AR expressed in oocytes treated with or without R1881 was partially affinity-purified using the FLAG-tag-specific M2 agarose beads to reduce the nonspecific DNA binding by oocyte extracts. As shown in Fig. 5, AR purified from both R1881 untreated or treated oocytes can bind to the ARE in a dose-dependent manner. No significant difference can be observed in terms of the binding (or protection) of the ARE sequence by both R1881 treated or untreated AR. Interestingly, the protection by R1881-treated AR appeared to extend more broadly than that by unliganded AR (compare lane 7 with 4). This difference may reflect the difference in conformations between liganded and unliganded AR and/or association of liganded AR with additional protein(s). Taken together, both gel mobility shift and DNase I footprinting assays demonstrate that AR binds to a consensus ARE in a ligand-independent manner, providing a crucial support for the observation of the ligand-independent activation.

AR Expressed in Oocytes Exists in a Protein Complex(es)

Because it is generally believed that in the absence of ligand AR in mammalian cells is associated with heat

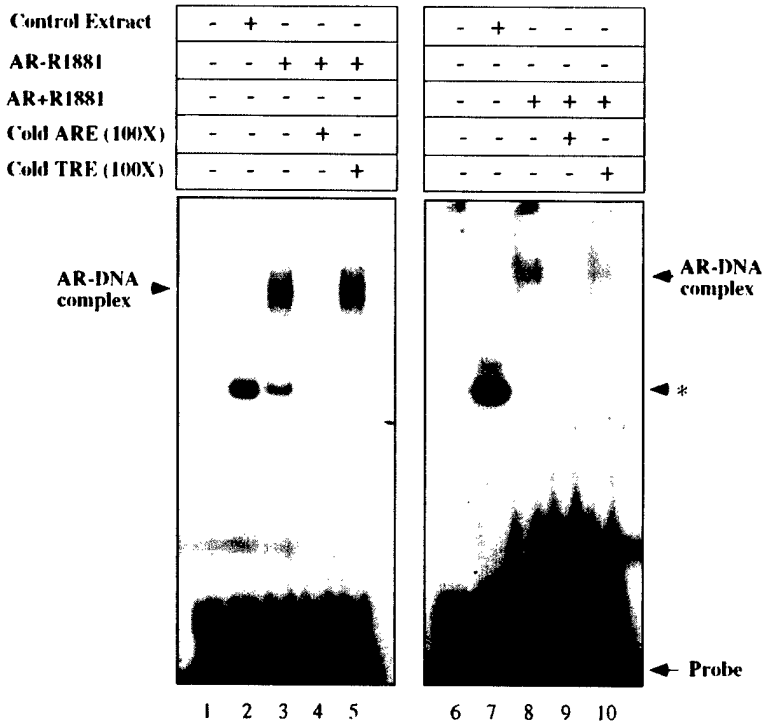

Fig. 4. AR Expressed in *Xenopus* Oocytes Binds to a Consensus ARE in a Ligand-Independent Manner

The extracts prepared from control oocytes or oocytes injected with AR mRNA (1 μg/μl, 18.4 nl/oocyte) and treated with or without R1881 (10 nM) were used for gel mobility shift assays. *, Nonspecific protein-DNA complex also present in the control oocytes. This nonspecific complex can be competed by addition of both ARE and TRE competitors. The position of AR-DNA complex is also indicated. Note that the AR-DNA complex is present only in the AR-containing extracts and can be competed out by addition of an excessive cold ARE but not the TRE competitor.

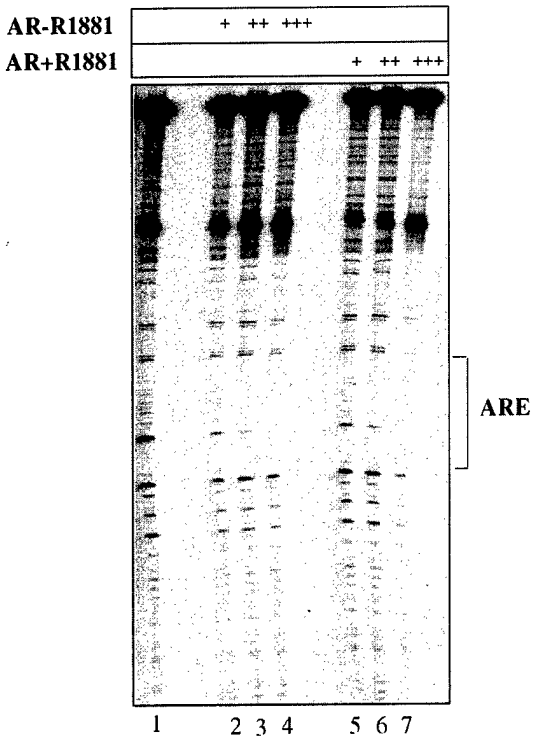

Fig. 5. DNase I Footprinting Assays Indicate that AR Binds in a Ligand-Independent Manner Specifically to the ARE

The end-labeled probe containing a consensus ARE sequence was generated by PCR. An increasing amount of partially purified AR (2 μl in lanes 2 and 5, 4 μl in lanes 3 and 6, and 8 μl in lanes 4 and 7) were used in the DNase I footprinting assay. The lane 1 is the control DNase I digestion without addition of partially purified AR. The position of the ARE is as indicated.

shock proteins (4), we next examined whether AR expressed in *Xenopus* oocytes is existed in protein complex(es). Toward this end, oocyte extracts derived from AR-expressing oocytes treated with or without R1881 were analyzed by gel filtration using a Superose 6 column. As shown by the Western analysis in Fig. 6A, the peak of the unliganded AR behaved as protein complex of 600–700 kDa (peak around fractions 22–24). Clearly, the peak of the AR from R1881 treated oocyte extracts shifted toward the right and thus appeared to be smaller (500 kDa) (peaks around fractions 26–28) (Fig. 6B). This result is consistent with the idea that binding of ligand induces the conformational change and/or dissociation of AR associated heat shock proteins or other protein(s). Nevertheless, when DNA binding activity was assayed by gel mobility shift across the fractions, both R1881 untreated and treated AR bound to the ARE as already demonstrated in Figs. 5 and 6. It is noteworthy that the DNA binding activity across the fractions from both R1881-untreated and -treated oocyte extracts correlated directly with the presence of AR, but not the sizes of the AR complex. This result is important because it rules out the possibility that the DNA binding activity from R1881 untreated AR containing extract is derived from a subfraction of AR proteins that may not be integrated into the protein complex(es) and thus presumably not associate with heat shock proteins. If dissociation of heat shock proteins is required for AR DNA binding, one would expect that the smaller AR complex migrating toward the right may be free of heat shock proteins and thus exhibit a better DNA binding capacity. However, our effort to check directly the presence of heat shock protein in AR complex(es) was hindered by the lack of antibody in our hands that can recognize heat shock proteins in *Xenopus* oocytes.

Coactivators Stimulate Both R1881-Dependent and -Independent Activation

Because the activity of the NRs is subject to regulation by coactivators, we next tested whether the hormone-independent activation by AR could be influenced by coactivators such as members of the SRC-1 family or p300. To better observe the effect of coactivators on ligand-independent activity of AR, we chose to express a moderate level of AR by injecting a medium concentration of AR mRNA (300 ng/μl). The expression of coactivators SRC-1, RAC3, or p300 was achieved by injection of their corresponding *in vitro* synthesized mRNA and confirmed by Western analysis (data not shown, see Ref. 39). As shown in Fig. 7A where the MMTV reporter was used, coexpression of SRC-1 and RAC3 with AR led to a significant enhancement of R1881-independent activation (from 4-fold to 16- and 17-fold, respectively). Under the same conditions, SRC-1 and RAC3 only moderately stimulated the transcription in the presence of R1881 (from 23-fold to 41- and 31-fold, respectively). The stimulation of R1881-independent activity by coactivators was not restricted to the MMTV reporter, as expression of p300 also stimulated the R1881-independent activation from the 4·ARE·TRβA-based reporter from 7- to 22-fold (Fig. 7B). As a control, expression of p300 alone (Fig. 7B, compare lane 2 with 1) or SRC-1 and RAC3 alone (data not shown) in the absence of AR did not stimulate transcription, indicating that the stimulation of transcription by those coactivators is mediated through AR. Thus, much like the hormone-dependent activation, the hormone-independent activation can be enhanced by the action of coactivators such as SRC1, RAC3, and p300.

Ligand-Independent Activation by AR Is Also Present in Mammalian Cells

To ascertain whether this ligand-independent activity by AR was unique to *Xenopus* oocytes, we also tested the ligand-independent activity of AR in mammalian cells by transient transfection. A luciferase reporter under the control of MMTV LTR was cotransfected with different amounts of an AR expression construct into COS-1 cells and treated with or without 10 nM of R1881. After 24 h incubation, cells were collected and processed for the luciferase assay. As shown in Fig. 8, although R1881-

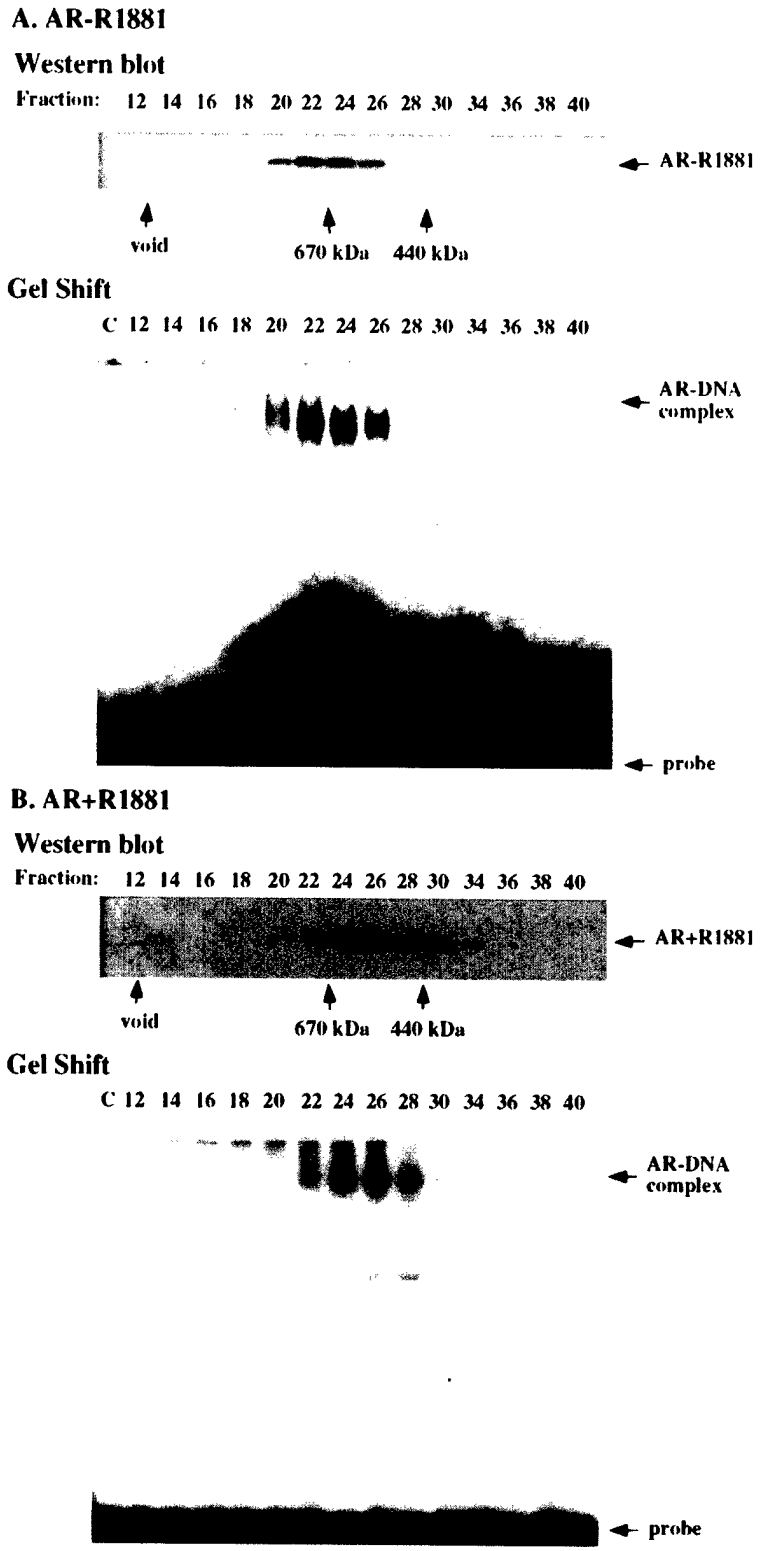

Fig. 6. AR Expressed in *Xenopus* Oocytes Exists as a Protein Complex(es)

A, Extracts prepared from oocytes injected with AR mRNA but not treated with R1881 were fractionated on a Superose 6 column and the presence of AR and the binding activity to the labeled ARE across the fractions were analyzed by Western blotting (*upper panel*) and gel mobility shift (*lower panel*). The *number on the top* of the Western blotting and gel shift results represent the number of fractions from the Superose 6 column. The *arrows at the bottom* indicate the elution positions of calibration proteins of known molecular weights. B, Same as panel A except that extracts were from the oocytes injected with AR mRNA and treated with R1881. Note that the peak fraction of AR from the sample not treated with R1881 appeared at fraction 24 and shifted to fraction 28 in the R1881-treated sample.

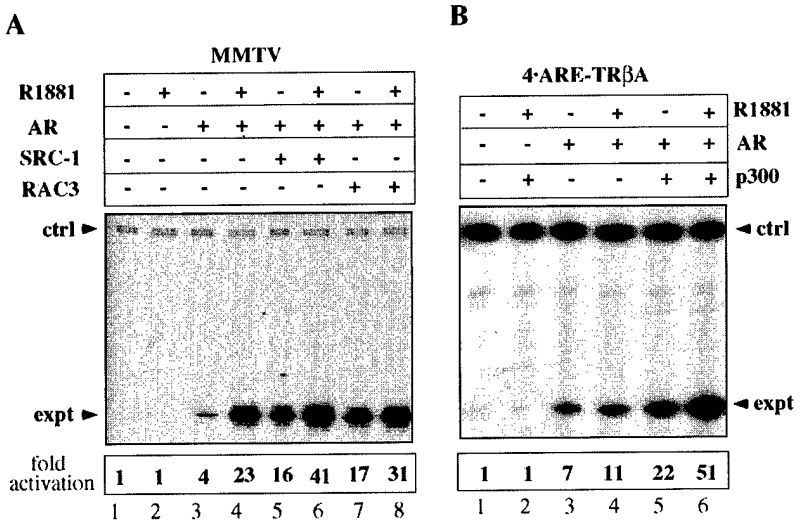

Fig. 7. Coactivators Such as SRC-1, RAC3, and p300 Stimulate Both Ligand-Dependent and -Independent Activation by AR

A, Both SRC-1 and RAC3 enhanced R1881-dependent and -independent activation by AR. Groups of oocytes were injected with a medium concentration of AR mRNA (300 ng/μl, 18.4 nl/oocyte) and mRNA encoding SRC-1 or RAC3 (100 ng/μl, 18.4 nl/oocyte) as indicated. The oocytes were then injected with ssDNA of the MMTV reporter and treated with or without R1881 (10 nM) overnight. The primer extension assay was as in Fig. 2. B, The coactivator p300 stimulates both R1881-dependent and -independent activation. The experiment was as in panel A except that mRNA encoding p300 and the 4·ARE-TRβA reporter were used.

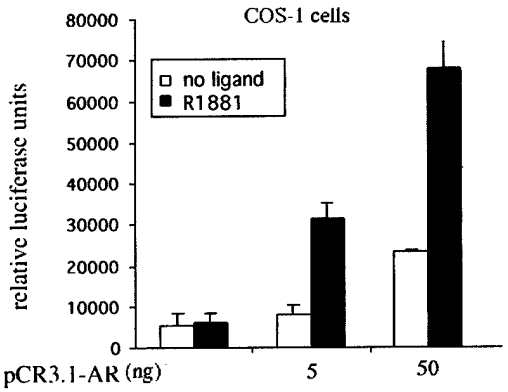

Fig. 8. Hormone-Independent Activation by AR Is Also Observed in Mammalian Cells

COS-1 cells were transiently transfected with MMTV-LTR-luc reporter and expression vector for AR as indicated. The luciferase data, expressed as relative light units, are the mean and SD of three independent transfection experiments.

independent activation by AR was not detectable when a low level of AR plasmid (5 ng) was used, the R1881-independent activation was clearly observed when a higher dose of AR expression plasmid (50 ng) was used. In both cases, addition of R1881 further stimulated the luciferase activity 4- and 3-fold, respectively. Thus, the ligand-independent activity by AR is not unique to *Xenopus* oocytes but likely an inherent feature of AR.

The Hormone-Independent Activity Can Be Inhibited by AR Antagonists

We next wished to test whether AR antagonists such as casodex and flutamide can inhibit the hormone-independent activity of AR. We first tested this in *Xenopus* oocytes. Groups of *Xenopus* oocytes were injected with the ssDNA of MMTV reporter, high dose of AR mRNA (1 μg/μl) and treated with either R1881 or antagonists as indicated (Fig. 9A). After overnight incubation, the levels of transcription were again determined by primer extension analysis. As expected, expression of AR led to a hormone-independent activation (Fig. 9A, compare lane 2 with 1). While addition of R1881 led to a further robust activation (Fig. 9A, compare lane 3 with 2), addition of either antagonists clearly inhibited the hormone-independent activation by AR (compare lanes 4, 5 and 6, 7 with 2).

We next tested in COS-1 cell whether antagonists could inhibit the hormone-independent activation. As shown in Fig. 9B, addition of antagonists casodex and flutamide indeed inhibited the hormone-independent activation by AR under the similar conditions as described in Fig. 8.

The Inhibition by Antagonist Correlates with the Recruitment of Corepressor SMRT

In an attempt to understand the mechanisms by which the antagonists inhibited the hormone-independent activity of AR, we analyzed whether casodex could influence the interaction of AR with coactivators and corepressors. We cotransfected a SRC-1 expression construct together with the AR expression construct into COS-1 cells. The transfected cells were then treated with or without R1881 or casodex as indicated (Fig. 9C). We then performed immunoprecipitation (IP) experiments using a FLAG-specific antibody (AR with a FLAG tag) and examined the co-IP of SRC-1. As shown in Fig. 9C,

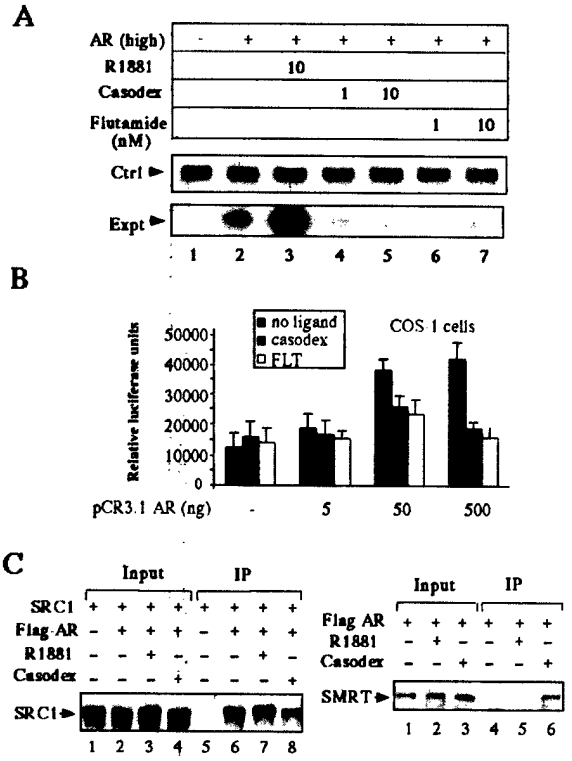

Fig. 9. Antagonists Can Inhibit Hormone-Independent Activation by AR and the Inhibition Is Correlated with the Reduction in Interaction of AR with Coactivator and Enhancement of the Association with Corepressor

A, Antagonists inhibit hormone-independent activation by AR in *Xenopus* oocytes. Groups of oocytes were injected with ssDNA of MMTV reporter (50 ng/μl, 18.4 nl/oocyte), AR mRNA at high concentration (1 μg/μl, 18.4 nl/oocyte) and treated with R1881, casodex and flutamide overnight at concentrations as indicated. The primer extension assay was as in Fig. 2. B, Antagonists also inhibit hormone-independent activation by AR in COS-1 cells. The transfection was similar to that in Fig. 8 except a higher amount of AR plasmid was also included. The concentration for both casodex and flutamide were 100 nM. C, Antagonist can modulate the interaction of AR with both coactivator and corepressor. A SRC-1 expression plasmid was cotransfected with FLAG-AR expression plasmid into COS-1 cells. IP was performed using a FLAG-tag-specific antibody. Western analyses were performed using a SRC-1-specific (*top*) or an SMRT-specific antibody (*bottom*) as indicated.

SRC-1 was co-IP with AR in the absence of hormone treatment (lane 6). Interestingly, addition of R1881 did not appear to have a significant effect the association of SRC-1 with AR (compare lane 7 with 6), whereas addition of casodex led to a slight reduction of the interaction of SRC-1 with AR (compare lane 8 with 6). Because several recent studies indicate that antagonists for estrogen receptors and progesterone receptors also have capacity to modulate interaction of corepressors SMRT and N-CoR with receptors (40, 41), we also tested whether casodex could induce interaction of AR proteins with corepressors such as SMRT and N-CoR. Western blotting using a SMRT-specific antibody revealed that addition of casodex resulted in co-IP of SMRT with AR (compare lane 6 with 4 at the right panel). Similar attempt using a N-CoR-specific antibody failed to detect N-CoR proteins in COS-1 cell extract, presumably because the level of N-CoR in COS-1 cells is low. Thus, the inhibitory effect of casodex appears to correlate with its ability to reduce the association of coactivator with AR as well as to enhance the recruitment of corepressor SMRT.

DISCUSSION

In the present study, we have reconstituted a R1881-responsive AR transcription system through microinjection of AR mRNA and reporters into *Xenopus* oocytes. We show that both the TRβA promoter and the MMTV-LTR-based reporters can be assembled into chromatin via the replication-coupled pathway through injection of the reporters in a ssDNA form (36). Addition of agonist R1881 leads to a robust activation from both reporters, whereas addition of the antagonists casodex and flutamide fails to do so. The establishment of this chromatin-based transcription system thus opens a new avenue for study of transcriptional regulation by AR in the context of chromatin. Indeed, we have evidence that activation from repressive chromatin by AR requires the involvement of coactivators and chromatin remodeling machinery (Huang, Z.-Q., and J. Wong, manuscript in preparation). The major findings from the work reported here are: 1) AR has a capacity to activate transcription in the absence of ligand (Figs. 3 and 8); 2) AR can bind to a consensus ARE *in vitro* in a hormone-independent manner (Figs. 4 and 5); 3) coactivators such as SRC-1, RAC3, and p300 stimulate both ligand-independent and -dependent activation by AR (Fig. 7) and 4) antagonists such as casodex can inhibit hormone-independent activation by AR and this inhibition appears to correlate with its ability to influence the association of AR with both coactivator and corepressor (Fig. 9).

While ligand-independent activation of AR by growth factors or other signaling pathways has been reported (24, 25), it is not clear whether AR itself has an intrinsic ligand-independent activity. By manipulating the levels of AR expression in oocytes through injection of different amounts of AR mRNAs, we demonstrate that high level expression of AR activated both the MMTV and TRβA-based reporters in the absence of R1881. Several lines of evidence support the conclusion that this hormone-independent activity is intrinsic to AR but not a unique feature of *Xenopus* oocytes. First, consistent with the observation from mammalian cells that AR proteins reside primarily in the cytoplasm, AR expressed in *Xenopus* oocytes also resides primarily in the cytoplasm in the absence of R1881. Second, AR expressed in *Xenopus* oocytes responds to agonist R1881 the same way as AR expressed in mammalian cells. These include the translocation from the cytoplasm to the nucleus and the

robust *trans*-activation of both reporters by AR in the presence of R1881. Third, high level expression can lead to the increase of nuclear AR. This is not surprising because the subcellular localization of AR is dynamic and likely to be influenced by its concentration. We believe that this unliganded AR in the nucleus is responsible for the observed ligand-independent transcription. Fourth, the *Xenopus* oocytes used here were not treated with growth factors or reagents that could activate PKA pathways. In other words, the R1881-independent activity of AR that we observed is unlikely a result of the activation of AR by cross-talk pathways. Nevertheless, we also could not rule out the remote possibility that a subpopulation of AR in *Xenopus* oocytes could be activated by other cross-talk signaling pathways or by mysterious ligand(s) in the oocytes. Fifth, overexpression of AR in COS-1 cells also leads to a R1881-independent *trans*-activation, indicating that the hormone-independent activation is not unique to *Xenopus* oocytes. Finally, as shown in several recent publications (19–21), coactivators such as members of SRC family interact with AR primarily through the AF1 but not the AF2 domain in AR. Consistent with those observations, we show that expression of SRC1, RAC3 and p300 in *Xenopus* oocytes further enhanced the ligand-independent activation by AR. Taken together, we propose that this hormone-independent transcriptional activity is intrinsic to AR and may be mediated through the hormone-independent interaction of AR with coactivators such as members of SRC family and p300.

Our demonstration that AR expressed in *Xenopus* oocytes exhibits ligand-independent DNA binding provides strong support for the idea that AR has the capacity to activate transcription in a ligand-independent manner. By both gel mobility and DNase I footprinting assays, we demonstrated that both unliganded AR and liganded AR bind to a consensus ARE. Furthermore, gel filtration analysis revealed that unliganded AR exists in a large protein complex(es) and that R1881 treatment causes AR to migrate as a smaller complex (Fig. 6). These results are consistent with the idea that in the absence of hormone AR is associated with other proteins including heat shock proteins and that binding of hormone results in the change of conformation and/or release of heat shock proteins. Nevertheless, gel mobility shift analysis of the gel filtration fractions derived from the R1881-untreated and -treated AR extracts indicates that DNA binding activity correlates with the presence of AR, not the size of the AR complex (Fig. 6). Taken together, these results provide strong evidence that AR can bind to an ARE in a ligand-independent manner. Ligand-independent DNA binding by AR has been reported before by using either *in vitro* translated AR proteins (31) or AR proteins expressed in insect SF9 cells (30). However, in many other cases treatment with ligand appears to be required for preparation of AR proteins with active DNA binding activity (33). This discrepancy could, at least in part, be explained by the technical difficulty in preparation of unliganded recombinant AR. AR expressed in SF9 cells is by and large insoluble in the absence of R1881 (30). On the other hand, R1881 treatment has been shown to induce AR expression due to the presence of AREs in the AR coding region and to stabilize AR proteins (42, 43). These two factors facilitate preparation of and the DNA binding assay for the liganded AR. Thus, the hormone-independent DNA binding activity is unlikely to be unique to the AR proteins expressed in *Xenopus* oocytes and may be an intrinsic feature of AR.

Our results that AR exhibits hormone-independent DNA binding and transcriptional activity also have strong implications for our understanding of the possible roles of AR in hormone-refractory prostate cancer. Strong evidence suggests that AR may remain functionally active and thus contribute to the progression of androgen-independent prostate cancer (44). Many androgen-independent prostate cancers are found to express both AR and its regulated genes (27–29). However, how AR remains transcriptionally active in androgen-independent prostate cancer is largely unknown. Many hypotheses, including mutations in AR, AR gene amplification, and protein overexpression; changes in coregulators; and activation of AR by cross-talk signaling pathways have been proposed. While mutations in AR may enhance activity of AR in the absence of ligand or a change in its hormone specificity, recent studies indicate that the frequency of AR mutations is low even in hormone-insensitive cancers (45, 46). Instead, the amplification and consequent overexpression of the wild-type AR gene appears to be the most common event found in hormone-refractory prostate cancers (45). These results suggest that overexpression of AR proteins is a potential mechanism that leads to the ligand-independent activity of AR in hormone-insensitive prostate cancer. Our results that overexpression of AR in *Xenopus* oocytes can result in R1881-independent activation of both TRβA promoter and MMTV LTR assembled into repressive chromatin provides support for this idea. Furthermore, comparison of AR in *Xenopus* oocytes injected with a high dose of AR mRNA with that in LNCaP cells indicates that a level of AR protein sufficient for observation of hormone-independent activity in *Xenopus* oocytes could be present in prostate cancer cells (Fig. 3C). In addition, we show that expression of coactivators such as members of the SRC family and p300 can further enhance the hormone-independent *trans*-activation by AR (Fig. 7). This result is consistent with the previous observation that AR could interact with and thus sequester SRC-1 protein even in the absence of hormone in mammalian cells (47). This result is also consistent with the recent reports that the AF1 but not the ligand-dependent AF2 domain of AR is primarily responsible for the interaction and recruitment of the SRC-1 family coactivators by AR (19–21). Given the ability to stimulate hormone-independent activation by AR, it is tempting to speculate that changes in levels of coactivators could be a

potential contributing factor for hormone-independent activation of AR in prostate cancer. One can envisage a scenario in prostate cancers in which gene amplification and overexpression of AR could result in hormone-independent activation of AR-regulated genes. The levels of this hormone-independent activation are likely to be further augmented by any increase in levels of coactivators. In addition, this hormone-independent activation could be further enhanced by cross-talk pathways mediated by growth factors (24, 25).

The finding that antagonists such as casodex can induce interaction of AR with corepressor SMRT is not surprising. It has been reported that antagonists for ERs and PRs can modulate interaction of corepressors SMRT and N-CoR with ER and PR (40, 41). Together, these findings indicate that, in addition to competing with agonists for binding of receptors, antagonists have capacity to actively repress the receptor activity by promoting their interaction with corepressor complexes.

In conclusion, the data presented here provide evidence that the interaction of AR with specific DNA sequences does not require ligand and that AR has the capacity to activate transcription in a ligand-independent manner when AR is overexpressed. This ligand-independent activity can be further enhanced by coactivators including the members of the SRC family and p300. It is of great interest to test in future whether this hormone-independent transcriptional activity is relevant to the occurrence and progression of androgen-independent prostate cancer.

MATERIALS AND METHODS

Plasmid Constructs

The 4·ARE-TRβA construct was generated by inserting four copies of the consensus ARE (AGAACC CCCTGTACC) into the *Nde*I site in the pTRβA-chloramphenicol acetyltransferase (CAT) gene construct (34). The ssDNA of the 4·ARE-TRβA construct was prepared from phagemids induced with helper phage VCS M13 as described (34). The MMTV-LTR-CAT construct was generated by inserting a fragment containing the MMTV LTR plus 0.3-kb CAT sequence into pBluescript II (SK^+), and the ssDNA was prepared as described (34). To produce *Xenopus* AR mRNA for microinjection, the cDNA encoding human AR with a FLAG-tag at the N terminus was subcloned into pSP64poly(A) vector. The pCR3.1-AR for expression of AR in mammalian cells was generated by subcloning AR cDNA into a modified pCR3.1 vector (Invitrogen, Carlsbad, CA) containing a N terminus FLAG tag. The MMTV-Luc reporter has been described previously (48). The plasmids for *in vitro* synthesis of SRC-1, RAC3, and p300 have also been described previously (39).

In Vitro mRNA Preparation and Microinjection of *Xenopus* Oocytes

To prepare AR mRNA *in vitro*, the pSP64poly(A)-AR was first digested with *Bgl*II. The synthesis of AR mRNA was carried out by using the linearized DNA template and a SP6 Message Machine kit (Ambion, Inc., Austin, TX) as described by the manufacturer. The *in vitro* synthesis of mRNAs encoding SRC-1, RAC3, and p300 was as described previously (39). A typical reaction with approximately 1 μg of linearized template in a 20 μl reaction yielded 10~15 μg of capped mRNA. All mRNAs were resuspended in ribonuclease-free water at a final concentration of 1 μg/μl. The preparation of stage VI *Xenopus* oocytes and microinjection were essentially as described (34). For transcriptional analysis, single-stranded reporter DNA was injected (50 ng/μl, 18.4 nl/oocyte) into the nuclei of the oocytes, whereas the indicated amount of mRNAs encoding AR or coactivators (100 ng/μl, 18.4 nl/oocyte) was injected into the cytoplasm of the oocytes. Injection of mRNAs was usually performed 2–3 h before the injection of ssDNA to allow protein synthesis. Usually a group of approximately 20 oocytes was injected for each sample to minimize variations among oocytes and injections. The injected oocytes were incubated at 18 C overnight in modified Barth's solution (36) supplemented with antibiotics (50 U/ml penicillin/streptomycin) in the presence or absence of 10 nM R1881 or the antagonists casodex and flutamide at concentrations indicated. The oocytes were then collected for transcription analyses or other assays as described below.

MNase Assay of Chromatin Structure

The MNase assay of chromatin assembly was performed as described previously (34).

Expression and Subcellular Localization of AR in Oocytes

To examine the expression and localization of AR in the oocytes, the cytoplasm and nucleus of the injected oocytes treated with or without R1881 (10 nM) were dissected manually. The protein extracts from cytoplasm, nucleus, and the whole oocytes were then resolved by SDS-PAGE followed by immunoblotting using an antibody against the FLAG-tag (1:5000 dilution). Signals were detected with a chemiluminescence kit (Pierce Chemical Co., Rockford, IL) as described by the manufacturer.

Transcription Analysis

Transcription analysis by primer extension was performed essentially as described (34). The primer I was used for detection of transcripts from the pTRβA and p4·ARE.TRβA reporters and CAT primer was used for detection of transcripts from the MMTV construct (34). The internal control was the primer extension product of the endogenous histone H4 mRNA using a H4-specific primer as described (49). In the figures where levels of transcription were presented, the levels of transcription were quantified by using phosphorimage analysis and were the average results of at least two independent experiments.

Gel Mobility Shift Assay

To examine the DNA binding activity of AR proteins expressed in *Xenopus* oocytes, groups of oocytes were injected with AR mRNA (1 μg/μl) and treated with or without R1881 (10 nM) overnight. The oocytes were then collected, rinsed once and homogenized in the extraction buffer (10 μl/oocytes) [20 mM HEPES (pH 7.9), 75 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 0.1% NP40, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride]. To maintain association of AR with R1881, a final concentration of 10 nM of R1881 was included in the extraction buffer for making extracts derived from R1881 treated oocytes. The clean extracts were obtained after centrifugation of crude extracts at 13,000 rpm for 20 min at 4 C to remove yolk proteins and lipids and used for gel shift assay. In brief, the oocyte extracts (1–2 μl) were preincubated with the binding buffer [HEPES (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 2 mM DTT, 0.1 mM EDTA, and 0.25 μg of polydeoxyinosine-deoxy-

cytidine] and with or without 10 nM of R1881 in a final volume of 14 μl for 15 min on ice. The end-labeled oligonucleotide probe containing a consensus ARE (0.1 ng) was added to each binding reaction and the mixture was incubated for 20 min at room temperature. In competition assays, unlabeled ARE or TRE (10 ng) was added into the reaction and incubated on ice with oocyte extracts for 15 min before the addition of the probe mixture. DNA-protein complexes were resolved on 5% polyacrylamide gels (80:1 of polyacrylamide/bisacrylamide) containing 0.5× TBE and revealed by autoradiography.

Gel Filtration Analysis of AR Complexes

A Superose 6 column (Amersham Pharmacia Biotech, Piscataway, NJ) was preequilibrated with the gel filtration buffer (20 mM HEPES, pH 7.8; 150 mM KCl; 1 mM DTT; 0.2 mM phenylmethylsulfonyl fluoride) at a flow-rate of 0.3 ml/min. Clean oocyte extracts (200 μl) prepared from AR mRNA injected *Xenopus* oocytes treated with or without R1881 (10 nM) were fractionated at a flow-rate of 0.3 ml/min. Samples (15 μl) from every other fraction (450 μl) were analyzed either by gel mobility shift for AR-DNA binding activity or by Western blotting for the presence of AR.

DNase I Footprinting

The DNase I footprinting assay was performed essentially as described (34) with after modifications. An end-labeled DNA fragment containing a consensus ARE was prepared by PCR, purified by PAGE and used for footprinting. The liganded AR and unliganded AR proteins used for footprinting assays were first partially affinity purified using the FLAG-tag specific M2 agarose resins (Sigma, St. Louis, MO) to reduce the nonspecific binding activity from oocyte extracts.

Cell Culture, Transient Transfection, Coimmunoprecipitation, and Western Blotting

LNCaP cells were culture in Roswell Park Memorial Institute 1640 medium (Invitrogen), which was supplemented with 5% FBS and glutamine. The whole cell extract of LNCaP cells was prepared by using the lysis buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 150 mM NaCl; and 0.5% NP40) followed by a centrifugation (14,000 rpm, 20 min at 4 C). COS-1 cells were cultured in DMEM with addition of 10% FBS. For luciferase assay, $1\sim2 \times 10^4$ COS-1 cells were plated in six-well plates in phenol red-free medium supplemented with 10% dextran charcoal-stripped FCS 24 h before transfection. Transient transfection was performed according to the protocol of the LipofectAMINE-plus kit (Life Technologies, Inc., Gaithersburg, MD), with addition of 100 ng of reporter MMTV-luc and the indicated amount of AR expression plasmid pCR3.1-AR for each well. After incubation for 16 h, the cells were washed and supplemented with fresh medium containing 10 nM R1881 or antagonists as indicated. After a further 24-h incubation, the cells were washed with cold PBS and lysed with the lysis buffer described above. The extracts were analyzed for luciferase activity according to a manufacturer's instruction (Promega Corp. luciferase assay kit) and the relative luciferase activity was normalized to the protein concentration. The results were the averages from at least three independent experiments. For coimmunoprecipitation experiments, expression constructs for FLAG-tagged AR and SRC-1 were cotransfected into COS-1 cells and treated with R1881 or antagonists as described above. The whole cell extracts were prepared and used for immunoprecipitation of AR using the FLAG-tag-specific antibody (M2, Sigma). The presence of SRC-1 or SMRT was detected by Western blotting using a SRC-1-specific antibody (39) and an SMRT-specific antibody (raised against amino acid 1165–1363 of human SMRT) (16). The AR antibody (N-20) for Western shown in Fig. 1A was purchased from Santa Cruz Biotechnology, Inc.

Acknowledgments

We thank Nancy Weigel for providing the antagonists casodex and flutamide. We are grateful to Austin Cooney, Neil McKenna, Nancy Weigel, David Rowley, and Bert W. O'Malley for critical reading of and comments on the manuscript.

Received August 29, 2001. Accepted January 10, 2002.

Address all correspondence and requests for reprints to: Dr. Jiemin Wong, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. E-mail: jwong@bcm.tmc.edu.

This work was supported in part by the Defense Prostate Cancer Program (PCRP) Idea Development Award PC991505 (to J.W.).

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The EMBO Journal Vol. 22 No. 9 pp. 2146–2155, 2003

A role for cofactor–cofactor and cofactor–histone interactions in targeting p300, SWI/SNF and Mediator for transcription

Zhi-Qing Huang, Jiwen Li, Laurent M.Sachs[1], Philip A.Cole[2] and Jiemin Wong[3]

Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, [2]Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA and [1]Département Régulation, Développement et Diversité Moléculaire, UMS 501 MNHN, UMR CNRS 8572, 7 rue Cuvier, 75231 Paris cedex 05, France

[3]Corresponding author
e-mail: jwong@bcm.tmc.edu

Transcriptional activation from chromatin by nuclear receptors (NRs) requires multiple cofactors including CBP/p300, SWI/SNF and Mediator. How NRs recruit these multiple cofactors is not clear. Here we show that activation by androgen receptor and thyroid hormone receptor is associated with the promoter targeting of SRC family members, p300, SWI/SNF and the Mediator complex. We show that recruitment of SWI/SNF leads to chromatin remodeling with altered DNA topology, and that both SWI/SNF and p300 histone acetylase activity are required for hormone-dependent activation. Importantly, we show that both the SWI/SNF and Mediator complexes can be targeted to chromatin by p300, which itself is recruited through interaction with SRC coactivators. Furthermore, histone acetylation by CBP/p300 facilitates the recruitment of SWI/SNF and Mediator. Thus, our data indicate that multiple cofactors required for activation are not all recruited through their direct interactions with NRs and underscore a role of cofactor–cofactor interaction and histone modification in coordinating the recruitment of multiple cofactors.

Keywords: chromatin remodeling/cofactor recruitment/NRs

Introduction

Initiation of transcription in eukaryotic cells is a complicated multistep process involving a large number of cofactors that exert functions in remodeling of chromatin and/or recruitment of RNA polymerase II (Pol II) to the promoters of target genes (Lemon and Tjian, 2000). Because packaging of eukaryotic DNA into chromatin has a generally repressive effect on transcription, enzymes that alter chromatin structure have critical roles in the regulation of gene expression (Narlikar *et al.*, 2002). The genomes of all eukaryotes studied code for a large number of chromatin remodeling and modification factors that are thought to evoke such changes (Jenuwein and Allis, 2001; Roth *et al.*, 2001; Becker and Horz, 2002). Indeed, genetic and functional evidence places ATPase-containing complexes such as SWI/SNF or PBAF upstream of gene activation and repression (Fryer and Archer, 1998; Goldmark *et al.*, 2000; Lemon *et al.*, 2001). Furthermore, a large number of enzymes that generate a wide spectrum of covalent modifications on chromatin and non-histone regulators are also known to be required for processes as diverse as mating type loci silencing in yeast and transcriptional activation in mammals (Jenuwein and Allis, 2001; Turner, 2002). In all cases, however, the structural nature of the requirement for these chromatin modifying and remodeling activities in generating the transcriptionally active or repressed state *in vivo* remains a matter of much uncertainty and debate.

The nuclear receptors (NRs) form a large family of ligand-regulated transcription factors and play key roles in animal development, differentiation, homeostasis and tumorigenesis (Mangelsdorf *et al.*, 1995). Transcriptional activation driven by liganded NRs has been associated with extensive chromatin structure alterations at target gene promoters and enhancers (Hager *et al.*, 2000; Urnov and Wolffe, 2001; Kraus and Wong, 2002). Strong evidence illuminates the involvement of histone acetyltransferases (HATs) such as CBP/p300, ATP-dependent chromatin remodeling complexes such as SWI/SNF or PBAF, and a complex (Mediator/TRAP/DRIP) that mediates communication with the basal transcriptional machinery in transcriptional activation by liganded NRs (Chakravarti *et al.*, 1996; Fondell *et al.*, 1996; Kamei *et al.*, 1996; Rachez *et al.*, 1998; Dilworth *et al.*, 2000; Lemon *et al.*, 2001). Whilst these activities are known to be targeted to NR-regulated promoters *in vivo* (Shang *et al.*, 2000; Sharma and Fondell, 2002), the mechanisms by which NRs recruit multiple cofactor complexes remain poorly defined.

One possibility is that NRs recruit each cofactor complex through a direct NR–cofactor interaction. In support of this model, NRs have been reported to interact directly with the components of SWI/SNF (Ichinose *et al.*, 1997; Nie *et al.*, 2000; Belandia *et al.*, 2002) and Mediator (Fondell *et al.*, 1996; Rachez *et al.*, 1998). Although CBP/p300 may interact directly with NRs, its participation in transcriptional activation by NRs is most likely mediated through interaction with SRC family coactivators (Li *et al.*, 2000; Sheppard *et al.*, 2001; Demarest *et al.*, 2002). The SRC family consists of three highly related and possibly functionally redundant proteins that interact with NRs in a hormone-dependent manner and will be referred to herein under the unified nomenclature SRC-1, SRC-2 and SRC-3 (McKenna *et al.*, 1999; Leo and Chen, 2000). Because SRC family coactivators, Mediator, and SWI/SNF all exist as large protein complexes and all appear to interact with a common binding site in the ligand-binding domain of the NRs, their association with a given NR molecule is thought to be mutually exclusive and is hypothesized to

occur in a step-by-step, iterative manner (Ito and Roeder, 2001). Interestingly, the 'order of recruitment'—if it exists—between the multitude of cofactors involved remains ill-defined, and appears to vary quite extensively between the very few cases where it has been studied *in vivo* (Cosma, 2002).

We present here a detailed *in vivo* analysis of molecular mechanisms by which well-studied representatives of both NR classes—the androgen receptor (AR; class I), and the thyroid hormone receptor (TR; class II)—induce activation in the context of chromatin. We show that hormone-dependent activation is associated with the specific recruitment of SRC family coactivators, p300, the SWI/SNF complex and the Mediator complex, to target gene promoters. We assay chromatin topology changes during activation to reveal the specific contribution that targeting of SWI/SNF makes to chromatin remodeling. We show that p300 has the capacity to mediate the recruitment of SWI/SNF as well as Mediator and that this recruitment is enhanced by histone acetylation exerted by CBP/p300. Our data suggest, therefore, that rather than proceed in a sequential manner by exchanging cofactors with NRs, all the remodeling, modification and Mediator complexes can be jointly recruited by the chromatin-bound NR via an adapter molecule (SRC) and that histone modification by one cofactor (p300) has a role in the recruitment of others (SWI/SNF and Mediator).

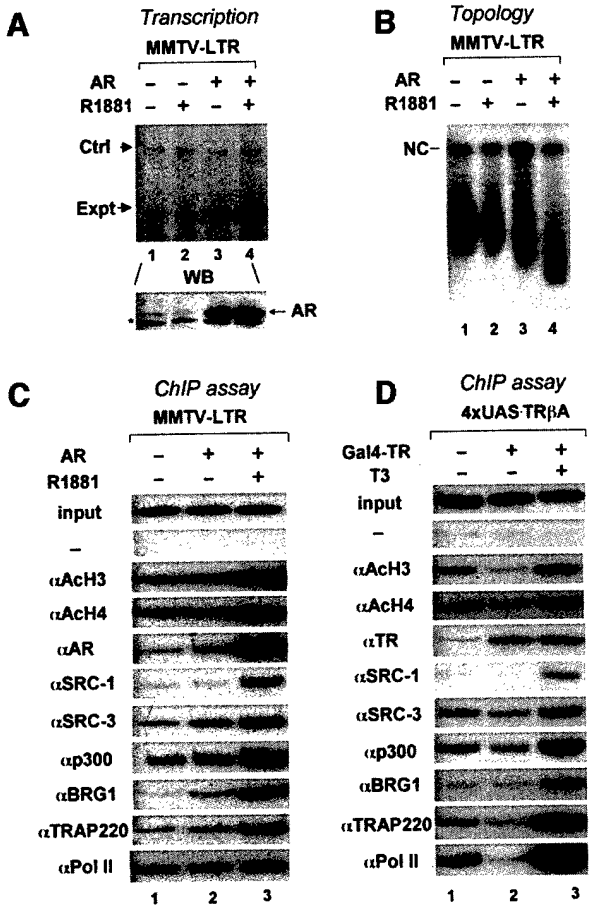

Fig. 1. Hormone-dependent activation by AR and TR is associated with chromatin remodeling with changes in DNA topology, histone acetylation and specific targeting of SRC-3, p300, BRG1 and TRAP220. (**A**) R1881-stimulated activation by AR from a MMTV-LTR reporter assembled into chromatin in *Xenopus* oocytes. The Ctrl represents the primer extension product from the oocyte storage histone H4 RNA. The expression of AR was detected by western blot using a FLAG-tag-specific antibody (M2; Sigma). (**B**) The same groups of oocytes as in (A) were analyzed for chromatin remodeling by a DNA topology assay. NC, nicked DNA. Faster migration in lane 4 in comparison with the control DNA indicates the loss of negative superhelical turns. (**C**) ChIP assays reveal histone acetylation and recruitment of different cofactors and RNA Pol II during R1881-stimulated activation by AR. The injection and treatment of oocytes were as in (A). Input DNA used for PCR was 5% of the total input DNA. Negative control (–) was performed without addition of antibody. (**D**) T3-dependent activation by TR is also associated with histone acetylation and promoter targeting of different cofactors and RNA Pol II. ChIP assays were performed with oocytes injected with 4×UAS.TRβA reporter and mRNA encoding Gal4–TR and treated with 50 nM T3 as described.

Results

Ligand-dependent activation by AR is associated with chromatin remodeling

Previously we have demonstrated that hormone-dependent activation by TR is associated with alterations in chromatin that can be detected as loss of canonical nucleosomal ladder revealed by partial micrococcal nuclease digestion or loss of negative superhelical turns by a DNA topology assay (Wong *et al.*, 1995, 1997). Recently we have reconstituted an agonist-responsive *Xenopus* oocyte transcription system for AR, a class I nuclear receptor, by assembling a MMTV-LTR-based reporter into chromatin via a replication-coupled pathway and expressing AR through microinjection of *in vitro* synthesized AR mRNA (Huang *et al.*, 2002). This allowed us to test whether alteration of chromatin structure is also associated with AR activation. The result (Figure 1A) showed that addition of R1881, a synthetic AR agonist, not only stimulated transcription, but also resulted in an extensive loss of negative superhelical density (Figure 1B). A small change in topology also could be seen in the absence of R1881 (Figure 1B, compare lane 3 with 1), most likely as a consequence of hormone-independent activity by AR (Huang *et al.*, 2002). The effect on DNA topology is not restricted to the MMTV-LTR reporter, as it was also observed when a different reporter was used (see Supplementary figure 1 available at *The EMBO Journal* Online). These experiments demonstrate that R1881-stimulated transcriptional activation is coupled with a chromatin remodeling event, as reported previously for TR (Wong *et al.*, 1995, 1997).

Hormone-dependent activation by AR and TR involves specific recruitment of both classes of chromatin remodeling factor and Mediator

We next wished to analyze the cofactors specifically recruited to the promoter region by AR during transcriptional activation using a chromatin immunoprecipitation (ChIP) assay. In brief, *Xenopus* oocytes were injected with AR mRNA and MMTV-LTR reporter and treated or not with R1881, and after overnight incubation, the chromatin-associated proteins were crosslinked by incubation of the oocytes with formaldehyde, fragmented by sonication and specific antibodies as indicated were used to precipitate DNA crosslinked to the target proteins. PCR was performed to measure the amount of MMTV-LTR

present in the immunoprecipitates. As shown in Figure 1C, R1881-stimulated activation is associated with the increased association of SRC-1, SRC-3, p300, BRG1 and TRAP220 and increased acetylation in histones H3 and H4. The increased association of BRG1, the ATPase subunit of the SWI/SNF complex and highly related PBAF complex (Lemon *et al.*, 2001) (for simplicity, we refer to SWI/SNF and PBAF collectively as SWI/SNF), and TRAP220, a subunit of the metazoan Mediator, suggests that both SWI/SNF and the Mediator complexes were recruited by liganded AR. Finally, the association of Pol II, as revealed by using a Pol II large subunit-specific antibody, was also increased during hormone-dependent activation.

In parallel, we also tested whether those cofactors were recruited by TR during T3-dependent activation. For convenience, we carried out the experiments using a fusion protein containing the Gal4 DNA-binding domain (DBD) and the TR ligand-binding domain and a TRβA promoter-based reporter bearing four Gal4-binding sites (4×UAS.TRβA) (Li *et al.*, 2002). Consistent with our previous results that unliganded TR repressed transcription as a result of specific recruitment of the HDAC3-containing SMRT/N–CoR complexes (Li *et al.*, 2002), deacetylation of histone H3 and H4 was observed in the absence but not in the presence of T3. Under the condition where activation by TR was observed, ChIP assays revealed the increased association of cofactors SRC-1, SRC-3, p300, BRG1, TRAP220 and RNA Pol II as well. Although the results in Figure 1D were obtained with addition of T3 overnight, a time-course experiment (Supplementary figure 2) showed a similar result and revealed no significant fluctuation in cofactor recruitment. Taking these results together, we conclude that transcriptional activation by both AR and TR is correlated with the recruitment of SRC family members, p300, SWI/SNF and the Mediator complex.

The recruitment of SWI/SNF, but not histone acetylation, is responsible for chromatin remodeling with change in DNA topology

The above results indicate that hormone-dependent activation by AR and TR is associated with targeted histone acetylation (Figure 1C and D) and alteration of DNA topology (Figure 1B; see Wong *et al.*, 1997). Whether these two events are interrelated is not clear. One possibility is that topology alteration during ligand-dependent activation is a consequence of chromatin acetylation. To test this possibility, we treated oocytes injected with the 4×UAS.TRβA reporter overnight with a potent HDAC inhibitor, trichostatin A (TSA). As expected, this treatment induced histone hyperacetylation as revealed by ChIP (Figure 2A). However, this marked hyperacetylation of chromatin did not lead to any

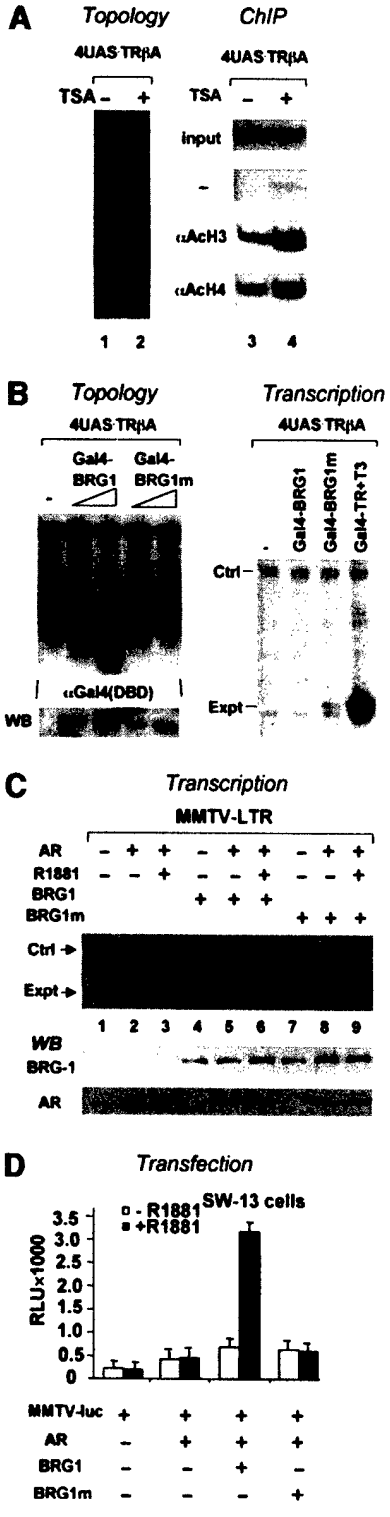

Fig. 2. Targeting BRG1 to chromatin is sufficient for chromatin remodeling with changes in DNA topology, and a functional BRG1 is required for AR activation. (**A**) Histone acetylation is not the reason for changes in DNA topology. (**B**) Targeting of a wild type BRG1 but not an ATPase-deficient mutant to chromatin resulted in chromatin remodeling with change in topology. Groups of oocytes were injected with mRNA encoding Gal4–BRG1 or Gal4–BRG1m (K785R) and 4×UAS.TRβA reporter as indicated. After overnight incubation, the oocytes were used for analysis of DNA topology by supercoiling assay (left panel) and protein expression by western blot (bottom panel). The right panel shows that Gal4–BRG1 did not activate transcription in comparison with Gal4–TR in the presence of T3. (**C**) Expression of wild-type BRG1 but not the mutant stimulated AR activation in *Xenopus* oocytes. The expression of AR and wild-type or mutant BRG1 was detected by western analysis using a FLAG-specific antibody (M2, Sigma). (**D**) R1881-dependent activation by AR was impaired in SW13 cells and could be restored by co-transfection of BRG1. SW13 cells were transfected with a MMTV-LTR-driven luciferase reporter and AR or BRG1 expression construct as indicated. All experiments were carried out in triplicate and repeated twice; error bars represent the SEM.

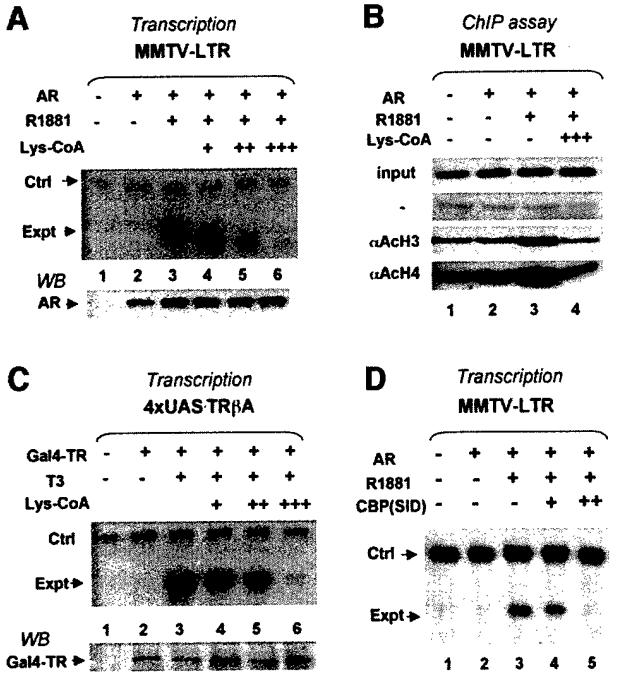

Fig. 3. Inhibition of CBP/p300 HAT activity or blocking SRC–CBP/p300 interaction abrogates hormone-dependent activation. (**A**) Endogenous CBP/p300 HAT activity is required for AR activation. The requirement of CBP/p300 HAT activity for AR activation was tested using a CBP/p300 selective HAT inhibitor, Lys-CoA. The oocytes were first injected with AR mRNA and then injected with ssDNA of the MMTV-LTR reporter (50 ng/μl, 18.4 nl/oocyte) containing 11.1 μM (+), 33.3 μM (++) or 100 μM (+++) of Lys-CoA. After overnight incubation with or without 50 nM R1881, the levels of transcription from the MMTV-LTR reporter were detected by primer extension assay and the level of AR protein is shown at the bottom panel by western blot. (**B**) ChIP assay revealed that Lys-CoA blocked hormone-dependent histone acetylation induced by AR. The groups of oocytes were injected with AR mRNA and ssDNA of the MMTV-LTR reporter with or without 100 μM of Lys-CoA as indicated. (**C**) Transcriptional activation by liganded TR also requires CBP/p300 HAT activity. (**D**) Expression of a CBP fragment (amino acids 2057–2170) containing the SRC interaction domain (SID) is sufficient to block AR activation.

measurable loss of superhelical density as compared with control chromatin (Figure 2A, compare lanes 1 and 2). These data exclude the possibility of histone acetylation as the sole cause of the DNA topology change observed during hormone-dependent activation.

Given the observed recruitment of BRG1 by liganded receptors (Figure 1C and D), the second possibility is that the observed topological change reflects chromatin remodeling by the SWI/SNF complex. To determine whether the SWI/SNF complex is responsible for the chromatin remodeling observed, we tested whether tethering to chromatin of the catalytic subunit of this complex, BGR1, which retains remodeling activity *in vitro* (Phelan *et al.*, 1999), is sufficient to induce chromatin remodeling *in vivo*. We used a chromatinized reporter bearing four Gal4 DNA-binding sites, and found that expression of a Gal4–BRG1 fusion protein in *Xenopus* oocytes caused chromatin remodeling to an extent comparable to that seen during NR-driven activation (Figure 2B). This chromatin remodeling is dependent on the level of Gal4–BRG1 expression, and, importantly, the ATPase activity of BRG1, because under the same experimental conditions, expression of a Gal4–BRG1 fusion carrying a point mutation in its ATPase domain failed to do so (Figure 2B). This data indicate that tethering BRG1 to chromatin phenocopies the chromatin remodeling event observed during ligand-dependent activation by AR and TR. Of note, no transcriptional activation was observed for Gal4–BRG1 (Figure 2B, right panel), indicating that chromatin remodeling by BRG1 or SWI/SNF itself is insufficient for transcriptional activation. Importantly, this chromatin remodeling appears to be specific for BRG1, as tethering a closely related ATPase, ISWI, to chromatin via Gal4 failed to evoke any degree of chromatin remodeling at any level of fusion protein expression (Supplementary figure 3A).

A requirement for SWI/SNF and CBP/p300 HAT in ligand-dependent transcriptional activation

To evaluate the roles of chromatin remodeling instigated by SWI/SNF, we first tested whether expression of BRG1 could enhance AR activation. Indeed, expression of BRG1 in *Xenopus* oocytes markedly stimulated the activation by liganded AR, whereas the expression of BRG1 alone had no effect on transcription (Figure 2C). Again, the ability to enhance AR activation appears to be specific to BRG1, as expression of ISWI failed to do so (Supplementary figure 3C). Importantly, expression of the K785R BRG1 mutant not only failed to stimulate but inhibited AR activation. We take this result to suggest that the mutant BRG1 inhibits AR activation by competing with the endogenous SWI/SNF required for activation by AR.

To further evaluate the role of the SWI/SNF complex in AR activation, we utilized a BRG1/Brm-deficient cell line, SW13, that is known to impair the function of retinoic acid receptor (RAR), glucocorticoid receptor (GR) and estrogen receptor (ER) (Muchardt and Yaniv, 1993; DiRenzo *et al.*, 2000). We found that transcriptional activation by AR was abrogated in SW13 cells, and that hormone-dependent activation could be rescued by introduction of an expression construct for wild-type BRG1 but not the remodeling-deficient K785R allelic version (Figure 2D). Thus, data both from *Xenopus* and mammalian cells provide strong evidence that BRG1 is required for hormone-dependent activation.

We next wished to determine the functional significance of histone acetylation in hormone-dependent transcriptional activation by AR and TR. As p300 was shown by ChIP assays to be recruited by both AR and TR, the observed histone acetylation most likely reflected the HAT activity of CBP/p300. We thus made use of a synthetic CBP/p300 selective HAT inhibitor, Lys-CoA, with an IC_{50} of ~0.5 μM for CBP/p300 versus 200 μM for PCAF (Lau *et al.*, 2000). The results (Figure 3A and C) showed that injection of this reagent inhibited both AR and TR activation in a dose-dependent manner. Given that the injection volume is <2% of the oocyte volume, the concentration of Lys-CoA in injected oocytes is expected to be diluted at least 50-fold, thus well below the concentration required for inhibition of PCAF activity. ChIP assays using acetylated H3- and H4-specific antibodies confirmed that at the concentration where the hormone-dependent activation was abrogated, targeted histone acetylation was also abolished (Figure 3B). We conclude that the HAT activity of CBP/p300 is essential

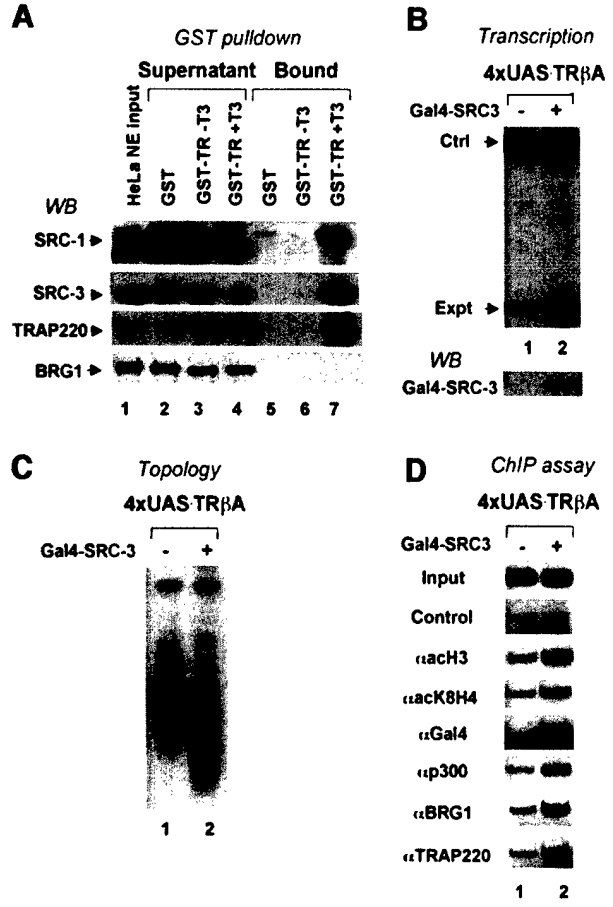

Fig. 4. The SWI/SNF complex is unlikely to be recruited directly by liganded TR but can be targeted to chromatin through a SRC family coactivator. (**A**) The interaction of SRC family coactivators, Mediator and SWI/SNF complex with TR was analyzed by GST–TR pulldown assay *in vitro*. Note that SRC-1, SRC-3 and TRAP220 were bound to GST–TR in a T3-dependent manner, whereas no interaction was observed between BRG1 and GST–TR. (**B**) Tethering SRC-3 to chromatin is sufficient for transcriptional activation. The oocytes were injected with or without mRNA encoding a Gal4–SRC-3 fusion protein (200 ng/μl, 18.4 nl/oocyte) and ssDNA of the 4×UAS.TRβA reporter. After overnight incubation, transcription was analyzed by primer extension in (B), DNA topology assay in (**C**) and ChIP assays in (**D**).

for hormone-dependent activation by AR and TR. As the targeted histone acetylation is also inhibited, our results imply that targeted histone acetylation plays a critical role in hormone-dependent activation by AR and TR, although the possibility that inhibition of non-histone substrate acetylation may also contribute to the inhibition of transcription can not be excluded.

SWI/SNF, p300 and the Mediator complex can be recruited to the NR via SRC family coactivators

Given the results (Figure 1C and D) that SRC coactivators, p300, SWI/SNF and Mediator are all targeted to promoter regions by liganded receptors, we next wished to examine how liganded receptors recruit these cofactors. The recruitment of CBP/p300, as shown previously (Li *et al.*, 2000; Sheppard *et al.*, 2001; Demarest *et al.*, 2002), is most likely mediated through its interaction with SRC family coactivators. In support, expression of a fragment containing the SRC-interaction domain of CBP (amino acids 2057–2170) in *Xenopus* oocytes effectively blocked transcriptional activation by AR (Figure 3D). As human Mediator was initially isolated as liganded TR-associated proteins (Fondell *et al.*, 1996), the recruitment of the Mediator complex can be explained as a ligand-dependent interaction between TRAP220 and receptors. However, despite numerous coimmunoprecipitation assays in mammalian cells or in *Xenopus* oocytes, we have yet to detect any ligand-dependent interaction between endogenous SWI/SNF or exogenously expressed BRG1 and either liganded TR or AR (data not shown).

We next used a simple assay to determine whether the SWI/SNF complex, which is abundant in HeLa nuclear extract, could interact directly with liganded NRs. We incubated HeLa nuclear extracts with a GST–TR fusion protein in the absence and presence of T3, and the proteins bound to the GST–TR were isolated and analyzed by western blotting. Significant quantities of endogenous SRC coactivators and of the Mediator complex, but no detectable SWI/SNF, associated directly with the liganded receptor in this assay (Figure 4A), suggesting that the recruitment of BRG1 by liganded NRs (Figure 1C–D) is most likely indirect.

Given the prominent interaction of SRC family coactivators with liganded receptors, we hypothesized that the SWI/SNF complex could be recruited indirectly through SRC family coactivators. We tested this hypothesis by determining whether tethering a SRC family coactivator to chromatin is sufficient to recruit SWI/SNF and to induce chromatin remodeling. The result in Figure 4B showed that expression of Gal4–SRC-3 in *Xenopus* oocytes activated transcription from the injected 4×UAS.TRβA reporter. Furthermore, a DNA topology assay (Figure 4C) showed that the expression of Gal4–SRC-3 also resulted in chromatin remodeling.

To understand how tethering a SRC coactivator is sufficient for transcriptional activation and chromatin remodeling, we performed ChIP assays (Figure 4D) to examine the recruitment of various cofactors and histone acetylation. Consistent with its role in recruitment of p300, ChIP assay revealed specific recruitment of p300 by Gal4–SRC-3 as well as acetylation of histones H3 and H4. Importantly, recruitment of BRG1 was also observed, thus demonstrating that a SRC family coactivator is capable of recruiting SWI/SNF through a direct or indirect interaction.

Consistent with our data showing that tethering of SRC is sufficient for transcriptional activation (Figure 4B), we found that Gal4–SRC-3 also recruited TRAP220, indicating that the Mediator complex can be recruited by a SRC family coactivator. The ability to activate transcription and induce chromatin remodeling as well as the recruitment of p300, BRG1 and TRAP220 is not restricted to SRC-3, as the same results were observed when SRC-1 and SRC-2 were tested under the same conditions (data not shown). These results indicate that members of the SRC coactivator family can function as an intermediary between liganded NRs and complexes critical for transcriptional activation such as CBP/p300, SWI/SNF, and Mediator.

The recruitment of SWI/SNF and Mediator is likely mediated through CBP/p300

Having established that SRC family coactivators have the capacity to recruit SWI/SNF and the Mediator complex,

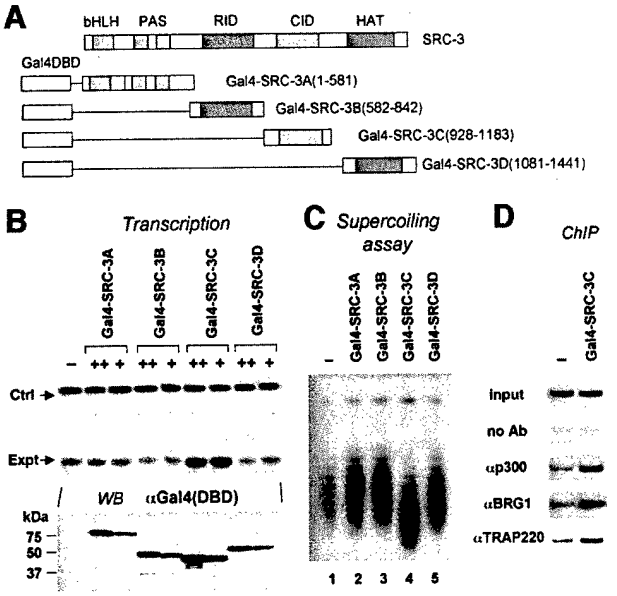

Fig. 5. The ability of SRC-3 to induce transcriptional activation, chromatin remodeling and recruitment of SWI/SNF and Mediator is correlated with its interaction with CBP/p300. (**A**) Diagram of the different functional domains of SRC-3 fused to Gal4 DBD. bHLH/PAS, basic helix–loop–helix and PAS dimerization domain; NID, nuclear receptor interaction domain; CID, CBP/p300 interaction domain; HAT, histone acetyltransferase domain. (**B**) Only the fragment containing the CID is sufficient for transcriptional activation when tethering to chromatin. The concentrations of mRNA used: +, 100 ng/μl and ++, 300 ng/μl. The transcription was assayed by primer extension and the protein expression was revealed by western analysis using a Gal4 DBD-specific antibody. (**C**) Topology assay showed that only Gal3-SRC-3C is capable of inducing DNA topological change. The concentration of mRNA used for each fusion protein is 300 ng/μl. (**D**) ChIP assays show that p300, BRG1 and TRAP220 can be recruited by Gal4–SRC-3C.

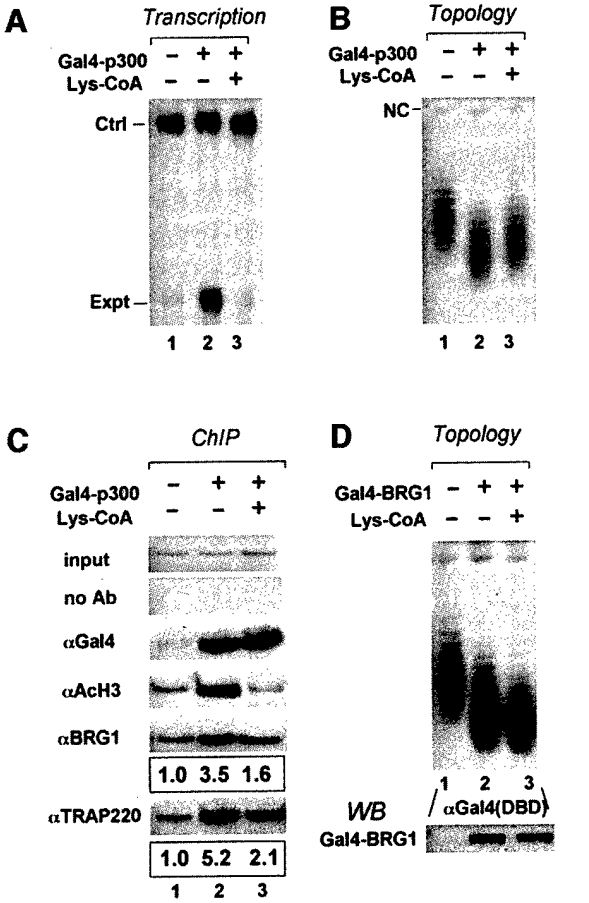

Fig. 6. SWI/SNF and Mediator complexes can be recruited by p300 and this recruitment is facilitated by histone acetylation. (**A**) Tethering of p300 to chromatin is sufficient for transcriptional activation and this activation can be inhibited by Lys-CoA. Oocytes were injected with Gal4–p300 mRNA (200 ng/μl) and ssDNA of the 4×UAS.TRβA reporter with or without addition of Lys-CoA and assayed for transcription after overnight incubation. (**B**) Injection was as in (A) but assayed for chromatin remodeling by DNA topology assay. Note that Lys-CoA partially inhibited loss of negative superhelical density. (**C**) ChIP assays revealed that SWI/SNF and Mediator can be recruited by Gal4–p300, and co-injection of Lys-CoA abolished histone acetylation and partially inhibited the recruitment of BRG1 and TRAP220. The results for BRG1 and TRAP220 were also analyzed by quantitative real-time PCR and the average values of three independent experiments are shown. (**D**) Lys-CoA has no effect on chromatin remodeling instigated by Gal4–BRG1.

we sought to determine which functional domain(s) in SRCs is required for this function. By using a series of SRC-3 constructs as shown in Figure 5A, we found that only the fusion protein containing the CBP/p300 interaction domain (CID) (Gal4–SRC-3C) significantly activated transcription from the 4×UAS.TRβA reporter (Figure 5B), whereas western analysis indicated that all fusion proteins were expressed at comparable levels (Figure 5B, lower panel). Furthermore, DNA topology assay indicated that only Gal4–SRC-3C induced a topological change (Figure 5C). Thus, both the transcriptional activation and chromatin remodeling functions of SRC-3 can be attributed to its CBP/p300 interaction domain. Indeed, side-by-side comparison of Gal4–SRC-3C and Gal4–SRC-3 revealed a similar capacity in transcriptional activation (Supplementary figure 4), suggesting that the CID represents the major, if not the entirety, of the autonomous activation activity of SRC-3.

To further understand the mechanism by which Gal4–SRC-3C activated transcription and induced chromatin remodeling, we carried out ChIP assays to verify the recruitment of p300, BRG1 and TRAP220. As shown in Figure 5D, expression of Gal4–SRC-3C indeed led to the recruitment of p300, BRG1 and TRAP220.

The above results indicate that the recruitment of SWI/SNF and the Mediator complex is most likely mediated through CBP/p300. To test this hypothesis, we tested transcription regulatory properties of a Gal4–p300 fusion protein. In full agreement with our expectation, expression of Gal4–p300 in *Xenopus* oocytes activated transcription from the reporter (Figure 6A) and induced chromatin remodeling (Figure 6B). Furthermore, ChIP assays confirmed the recruitment of BRG1 and TRAP220 by Gal4–p300 (Figure 6C). As expected, expression of Gal4–p300 also led to increased histone acetylation (Figure 6C, αacH3).

Functional interplay between histone acetylation and chromatin remodeling by SWI/SNF

As the above result points to CBP/p300 as the molecule that mediates SWI/SNF and Mediator recruitment, we assessed the potential role of histone acetylation in recruitment of SWI/SNF and Mediator. As shown in Figure 6A, activation by Gal4–p300 was abolished when its HAT activity was inhibited by the presence of

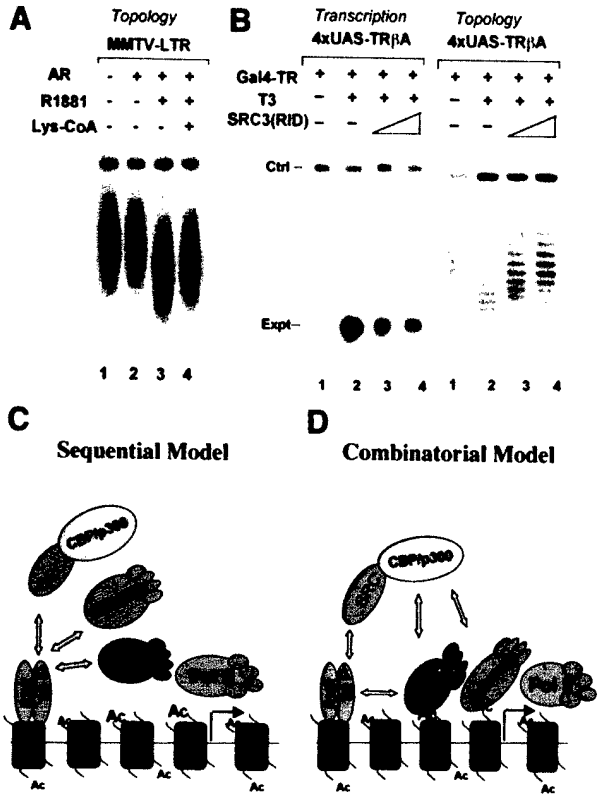

Fig. 7. Chromatin remodeling by liganded receptors is also partially dependent on histone acetylation, and blocking the recruitment of SRC coactivators inhibited chromatin remodeling. (**A**) Injection was similar to experiments in Figure 1A except that Lys-CoA (100 μM) was included as indicated. Note that Lys-CoA partially inhibited chromatin remodeling induced by liganded AR. (**B**) Expression of the RID of SRC-3 (amino acids 582–843) inhibited T3-dependent activation (left panel) and chromatin remodeling (right panel). (**C**) A sequential model showing that recruitment of SRC/p300, SWI/SNF and Mediator is achieved through independent interaction with liganded NRs. (**D**) A new combinatorial model showing that, in addition to NR–cofactor interaction, cofactor–cofactor and cofactor–histone interaction also contribute to the recruitment of multiple cofactors required for NR activation. CBP/p300 can mediate the recruitment of SWI/SNF and Mediator. Furthermore, histone acetylation by CBP/p300 facilitates subsequent recruitment of SWI/SNF and Mediator.

Lys-CoA. Under the same conditions, injection of Lys-CoA partially inhibited chromatin remodeling by SWI/SNF, as the loss of superhelical density was reduced (Figure 6B, compare lanes 3 and 2). Interestingly, ChIP assays revealed that, in the presence of Lys-CoA, the recruitment of TRAP220 and BRG1 was also partially reduced (Figure 6C). To assess whether Lys-CoA or histone acetylation had a direct effect on chromatin remodeling activity by SWI/SNF, we tested the effect of Lys-CoA on the chromatin remodeling induced by Gal4–BRG1. As shown in Figure 6D, the chromatin remodeling induced by Gal4–BRG1 was not affected by addition of Lys-CoA. This result suggests that histone acetylation enhances the recruitment (or stable association) of SWI/SNF during AR and TR activation, but histone acetylation itself is not required for BRG1- or SWI/SNF-induced chromatin remodeling.

Given the observation that recruitment of SWI/SNF by Gal4–p300 was partially dependent on histone acetylation (Figure 6C), we next wished to determine whether the chromatin remodeling induced by SWI/SNF during hormone-dependent activation is also partially dependent on histone acetylation. Indeed, the chromatin remodeling induced by liganded AR was partially inhibited by injection of the Lys-CoA inhibitor (Figure 7A). This result indicates that, as in the case of Gal4–p300, the recruitment of SWI/SNF by liganded AR is partially dependent on histone acetylation.

Blocking the recruitment of SRC coactivators inhibits chromatin remodeling induced by liganded receptors

The above results suggest a working model in which SWI/SNF is recruited by liganded receptors indirectly through the SRC/p300 cascade. A prediction of this model is that blocking the recruitment of SRC coactivators by liganded receptors would also block the recruitment of SWI/SNF. To our satisfaction, expression of the SRC-3 receptor interaction domain (amino acids 582–843) in *Xenopus* oocytes effectively blocked the DNA topological change instigated by SWI/SNF during activation by Gal4–TR (Figure 7B, right panel). As expected, this dominant-negative SRC-3 also inhibited T3-dependent transcription activation (Figure 7B, left panel).

Discussion

Chromatin remodeling by the SWI/SNF complex

Previous studies have correlated hormone-dependent transcriptional activation with chromatin remodeling, often revealed by loss of canonical nucleosomal ladder, formation of DNase I-hypersensitive sites, increased nuclease accessibility or changes in DNA topology. (Wong *et al.*, 1995, 1997; Bhattacharyya *et al.*, 1997; Fryer and Archer, 1998). Given the presence of multiple chromatin remodeling factors, it has been difficult to pinpoint the specific effect and function of a single factor. Here we provide compelling evidence that chromatin remodeling with change in DNA topology observed during hormone-dependent activation is instigated by the SWI/SNF complex targeted to chromatin by liganded receptors. First, as shown in Figure 2A, alteration in DNA topology is distinguishable from targeted histone acetylation and is not a consequence of histone acetylation. Secondly, as revealed by ChIP assays, BRG1, the ATPase subunit of the SWI/SNF complex, is recruited to the promoter regions by both liganded AR and TR. Thirdly, the chromatin remodeling with changes in DNA topology is recapitulated by tethering BRG1 but not the ISWI protein to chromatin (Figure 2B; Supplementary figure 3). These data are consistent with an *in vitro* study showing that only chromatin remodeling instigated by BRG1, but not by ISWI, is functionally coupled to changes in DNA topology (Aalfs *et al.*, 2001). Finally, expression of BRG1 stimulates transcription activation in an ATPase-dependent manner (Figure 2C) and a functional BRG1 is required for hormone-dependent activation (Figure 2D). Collectively our data establish SWI/SNF as responsible for chromatin remodeling with change of DNA topology observed during the hormone-dependent activation process. Furthermore, as chromatin remodeling with changes in DNA topology is closely related to the loss of canonical nucleosomal ladder and formation of DNase I-hypersensitive

sites (Wong *et al.*, 1997), it is tempting to suggest that the latter two assays also reflect the chromatin remodeling instigated by SWI/SNF.

Requirement for HAT activity of both CBP/p300 and SWI/SNF complex for hormone-dependent activation

Through exogenous expression of CBP/p300 mutants defective in HAT activity, previous work indicates that the HAT activity of CBP/p300 is required for its ability to function as coactivator for most, but not all, NRs (Korzus *et al.*, 1998; Kraus *et al.*, 1999; Li *et al.*, 2000). By using a CBP/p300 selective HAT inhibitor, Lys-CoA, we show that hormone-dependent activation by both AR and TR is critically dependent on endogenous CBP/p300 HAT activity. In addition, hormone-dependent activation by AR is also dependent on the function of SWI/SNF (Figure 2D), in agreement with results from other laboratories (Muchardt and Yaniv, 1993; Fryer and Archer, 1998; DiRenzo *et al.*, 2000). These data collectively indicate that histone acetylation by CBP/p300 and the chromatin remodeling by SWI/SNF are both critically important for hormone-dependent activation by NRs.

Although it is not yet clear why hormone-dependent activation by NRs requires both histone acetylation by CBP/p300 and chromatin remodeling by SWI/SNF, it is clear that histone acetylation and chromatin remodeling by SWI/SNF have distinct effects on chromatin. Only chromatin remodeling by SWI/SNF leads to the change of DNA topology (Figure 2), whereas histone acetylation *per se* fails to do so (Figure 2A). It is generally believed that histone acetylation is likely to affect nucleosomal higher order structure, histone tail–DNA interactions and/or serve as a 'code' for docking of regulatory proteins (such as SWI/SNF) and the basal transcriptional machinery (Wolffe and Hayes, 1999; Jenuwein and Allis, 2001; Roth *et al.*, 2001). In contrast, SWI/SNF can alter chromatin structure by inducing changes in conformation and/or position (sliding) and DNA topology, which is likely required for exposing important regulatory DNA sequences to transcription factors and especially basal transcription machinery (Kingston and Narlikar, 1999; Peterson and Workman, 2000; Becker and Horz, 2002). However, it should be noted that whether the requirement for both histone acetylation by CBP/p300 and chromatin remodeling by SWI/SNF is a general phenomenon for transcription activation from chromatin or is restricted to only a subset of genes such as targets for NRs is currently not known.

The roles of cofactor–cofactor interaction and histone modification in recruitment of CBP/p300, SWI/SNF and Mediator by liganded NRs

In addition to the NR–cofactor interaction identified previously, our study reveals a critical role of cofactor–cofactor interaction and histone modification in recruiting multiple cofactors whose concerted function is required for transcriptional activation by NRs. The role of SRC coactivators in recruiting CBP/p300 is well documented. Although previous studies suggest that NRs may recruit the SWI/SNF complex directly (Ichinose *et al.*, 1997; DiRenzo *et al.*, 2000; Nie *et al.*, 2000; Belandia *et al.*, 2002), our results indicate that SWI/SNF is unlikely to be recruited through a direct interaction with liganded TR (Figure 4A). Rather, on the basis of both functional and recruitment assays, we show that SWI/SNF can be recruited by NRs indirectly through p300, which itself is recruited through interaction with SRC coactivators (Figures 4–7). Furthermore, the recruitment of SWI/SNF is partially dependent on histone acetylation, as inhibition of histone acetylation by Lys-CoA partially inhibits the recruitment of SWI/SNF (Figure 6C). This result is consistent with the histone code hypothesis and the results that acetylated histones tails serve as docking sites for bromodomain-containing proteins. Our data are also consistent with recent *in vitro* and *in vivo* studies showing that histone acetylation stabilizes the recruitment of the SWI/SNF complex to chromatin and that H4 K8 acetylation serves for recruitment of SWI/SNF (Agalioti *et al.*, 2002; Hassan *et al.*, 2002). However, it should be noted that histone acetylation itself appears to be insufficient for recruitment of the SWI/SNF complex, since hyperacetylation induced by TSA treatment failed to induce a change in DNA topology (Figure 2A) or recruit SWI/SNF complex (data not shown). Rather, a direct or indirect interaction between p300 and SWI/SNF is essential and sufficient for targeting of SWI/SNF complex to promoter regions, because the recruitment of SWI/SNF by p300 is only partially inhibited under the condition where histone acetylation is abolished (Figure 6C). Thus, the recruitment of SWI/SNF is likely to be initiated through a direct or indirect interaction with CBP/p300, and histone acetylation exerted by CBP/p300 subsequently provide additional anchors to further stabilize the recruitment of SWI/SNF.

Numerous published data have clearly established a direct interaction between Mediator and NRs (Ito and Roeder, 2001; Baek *et al.*, 2002). Our result that tethering SRCs or p300 to chromatin is sufficient for the recruitment of the Mediator complex (Figures 4–7) also reveals an alternative pathway for recruitment of Mediator. This NR-independent recruitment may provide an explanation of why hormone-dependent activation by NRs is impaired but not abolished in TRAP220$^{-/-}$ mouse embryonic fibroblast cells (Ito *et al.*, 2000). Given the critical roles of the metazoan Mediator in both basal and activated transcription (Holstege *et al.*, 1998; Baek *et al.*, 2002), we suggest that the mild effect of TRAP220 deficiency on transcriptional activation by NRs could be explained by its indirect recruitment through CBP/p300. Interestingly, the recruitment of TRAP220 by p300 is also partially dependent on histone acetylation (Figure 6C), a result consistent with a recent study on the recruitment of Mediator complex to a TR-responsive gene in HeLa cells (Sharma and Fondell, 2002).

Together our data provide evidence for a new working model (Figure 7D) in which all cofactors essential for hormone-dependent activation by NRs can be recruited through a combinatorial effect of (direct or indirect) NR–cofactor, cofactor–cofactor and cofactor–histone interactions. In divergence from the sequential model in which different cofactors are all recruited through a direct interaction with liganded NRs (Figure 7C), in this new model, liganded NRs such as TR/RXR first interact with the members of SRC family coactivators, which in turn recruit CBP/p300. The recruitment of SWI/SNF is mediated through a direct or indirect interaction with

CBP/p300 and is further stabilized by histone acetylation exerted by CBP/p300. The Mediator complex can be recruited either through a direct interaction with NRs or indirectly through CBP/p300, thus revealing a role of histone acetylation by one factor (p300) in recruitment of other essential cofactors (SWI/SNF and Mediator). However, we shall point out that the models in Figure 7C and D are not mutually exclusive and that SWI/SNF can be potentially recruited directly by other NRs such as GR and ER. Given the wide involvement of CBP/p300 in gene expression, our data that CBP/p300 can mediate the recruitment of SWI/SNF and Mediator is of general significance in understanding transcription regulation in higher eukaryotes.

Materials and methods

Plasmid constructs

The reporter plasmids 4×UAS.TRβA and MMTV-LTR–CAT were as described previously (Huang *et al.*, 2002; Li *et al.*, 2002), respectively. The single strand (ss) reporter plasmids were prepared from phagemids induced with helper phage VCS M13 as described previously (Wong *et al.*, 1995). The constructs for *in vitro* synthesis of mRNAs encoding FLAG–SRC-1, FLAG–SRC-2, FLAG–SRC-3, p300, p300HATm, FLAG–AR and Gal4–TRβA were described previously (Li *et al.*, 2000; Huang *et al.*, 2002). The constructs for Gal4 fusions of SRCs, SRC-3 deletion mutants, BRG1 and BRG1(R785K), *Xenopus* ISWI and p300, respectively, were generated by inserting the corresponding cDNA fragments into the pSP64poly(A)-Gal4(DBD) vector.

In vitro synthesis of mRNAs and microinjection of Xenopus oocytes

The synthesis of mRNAs *in vitro* was carried out using DNA templates linearized with a suitable restriction enzyme and a SP6 Message Machine kit (Ambion, Inc.) as described by the manufacturer. The preparation of stage VI *Xenopus* oocytes and microinjection were essentially as described by Wong *et al.* (1995). Unless indicated in figure legends, ssDNA was injected (50 ng/μl, 18.4 nl/oocyte) into the nuclei of the oocytes, whereas the indicated amount of mRNAs encoding receptors or coactivators was injected into the cytoplasm of the oocytes (18.4 nl/oocyte). After overnight incubation with or without hormone, the oocytes were collected and divided into four groups for western analysis, transcription analysis by primer extension, DNA supercoiling assay and ChIP assays.

Supercoiling assay of chromatin structure, transcription analysis and western analysis

Analysis of DNA topology by supercoiling assay and transcription by primer extension were performed as described previously (Wong *et al.*, 1997; Huang *et al.*, 2002). The internal control was the primer extension product of the endogenous histone H4 mRNA using a H4-specific primer as described (Wong *et al.*, 1997). The analysis of protein expression in *Xenopus* oocytes after injection of mRNAs was carried out by western analysis using a FLAG-tag-specific antibody (M2, Sigma) or a Gal4 DBD-specific antibody (RK5C1; Santa Cruz Biotechnology) according to the manufacturer's instructions.

ChIP assays

ChIP assays with injected *Xenopus* oocytes were performed essentially as described (Li *et al.*, 2002). The ChIP PCR for the MMTV-LTR construct generated a 150 bp PCR product, whereas the reaction for 4×UAS.TRβA construct generated a product of 100 bp. The antibodies against acetylated H3 (06-599) or H4 (06-598) were purchased from Upstate Biotechnology. The antibodies against human SRC-1 and SRC-3 were generated as described previously (Li *et al.*, 2000) and recognized their *Xenopus* counterparts as well. The rabbit polyclonal antibody against *Xenopus* p300 was generated and affinity-purified against two synthetic peptides (amino acids 1033–1049 KSEPVELEEKKEEVKTE and amino acids 1487–1506 KPKRLQEWYKKMLDKSVSER). The BRG1 antibody was kindly provided by Dr Weidong Wang (NIA/NIH). The TRAP220 antibody was generated against a fragment of *Xenopus* TRAP220 (equivalent to human TRAP220 1351–1580).

Cell lines and transient transfection

The SW-13 adrenal carcinoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS; Sigma). Transient transfection and luciferase assay were essentially as described (Huang *et al.*, 2002).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Drs Fyodor D.Urnov, Sharon Dent and members of the Wong laboratory for critical reading and comments on the manuscript. We are grateful to Weidong Wang for BRG1 antibody and Paul Wade for *Xenopus* ISWI cDNA. This work is supported by DK56324 from NIH and PC991505 from the Department of Defense Prostate Cancer Program to J.W.

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Received December 12, 2002; revised March 5, 2003; accepted March 13, 2003

0888-8809/03/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 17(5):908–922

doi: 10.1210/me.2002-0308

Transcriptional Activation by Thyroid Hormone Receptor-β Involves Chromatin Remodeling, Histone Acetylation, and Synergistic Stimulation by p300 and Steroid Receptor Coactivators

KATHLEEN C. LEE*, JIWEN LI*, PHILIP A. COLE, JIEMIN WONG†, AND W. LEE KRAUS†

Department of Molecular Biology and Genetics (K.C.L., W.L.K.), Cornell University, Ithaca, New York 14853; Department of Molecular and Cellular Biology (J.L., J.W.), Baylor College of Medicine, Houston, Texas 77030; Department of Pharmacology and Molecular Sciences (P.A.C.), The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and Department of Pharmacology (W.L.K.), Weill Medical College of Cornell University, New York, New York 10021

Transcriptional regulation by heterodimers of thyroid hormone receptor (TR) and the 9-*cis* retinoid X receptor (RXR) is a highly complex process involving a large number of accessory factors, as well as chromatin remodeling. We have used a biochemical approach, including an *in vitro* chromatin assembly and transcription system that accurately recapitulates ligand- and activation function (AF)-2-dependent transcriptional activation by TRβ/RXRα heterodimers, as well as *in vitro* chromatin immunoprecipitation assays, to study the mechanisms of TRβ-mediated transcription with chromatin templates. Using this approach, we show that chromatin is required for robust ligand-dependent activation by TRβ. We also show that the binding of liganded TRβ to chromatin induces promoter-proximal chromatin remodeling and histone acetylation, and that histone acetylation is correlated with increased TRβ- dependent transcription. Additionally, we find that steroid receptor coactivators (SRCs) and p300 function synergistically to stimulate TRβ-dependent transcription, with multiple functional domains of p300 contributing to its coactivator activity with TRβ. A major conclusion from our experiments is that the primary role of the SRC proteins is to recruit p300/cAMP response element binding protein-binding protein to hormone-regulated promoters. Together, our results suggest a multiple step pathway for transcriptional regulation by liganded TRβ, including chromatin remodeling, recruitment of coactivators, targeted histone acetylation, and recruitment of the RNA polymerase II transcriptional machinery. Our studies highlight the functional importance of chromatin in transcriptional control and further define the molecular mechanisms by which the SRC and p300 coactivators facilitate transcriptional activation by liganded TRβ. (*Molecular Endocrinology* 17: 908–922, 2003)

THE MOLECULAR ACTIONS of thyroid hormone (T_3) are mediated through thyroid hormone receptors (TRα and TRβ). TRs belong to the nuclear receptor (NR) superfamily and play important roles in development, differentiation, homeostasis, and tumorigenesis through their ability to regulate gene expression (1). TRs function as heterodimers with the 9-*cis* retinoic acid receptor (RXR) and, in the absence of hormone, the heterodimers bind to thyroid hormone response elements (TREs) and actively repress transcription (2, 3). In contrast to unliganded TRs, ligand-bound TRs function as transcriptional activators. Ligand-dependent activation by TR/RXR heterodimers requires an intact activation domain residing at the carboxyl terminus of the TR ligand-binding domain [known as activation function (AF)-2], as well as cellular coactivators (4).

Many coactivators have been implicated in T_3-dependent activation, including the steroid receptor coactivator (SRC) family of proteins, p300/cAMP response element binding protein-binding protein (CBP), p300/CBP associated factor (PCAF), and the mediator-like TR associated proteins (TRAP)/vitamin D receptor-interacting proteins (DRIP)/SRB/mediator-containing cofactor complex (SMCC) (hereafter referred to as the TRAP complex; for reviews, see Refs. 5–9). The SRC family contains three highly related and possibly functionally redundant proteins referred to herein under the unified nomenclature SRC-1, SRC-2, and SRC-3 (5). The

Abbreviations: AF, Activation function; AT, acetyltransferase; CBP, cAMP binding protein-binding protein; CH, cys/his; ChIP, chromatin immunoprecipitation; CoA, coenzyme A; ER, estrogen receptor; DNase, deoxyribonuclease; DRIP, vitamin D receptor-interacting proteins; GST, glutathione-*S*-transferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; MNase, micrococcal nuclease; NR, nuclear receptor; PID, p300/CBP interaction domain; RID, receptor interaction domain; RXR, 9-*cis* retinoic acid receptor; SID, SRC interaction domain; SRC, steroid receptor coactivator; TK, thymidine kinase; TR, thyroid hormone receptor; TRβm, transcriptionally inactive xTRβ mutant; TRAP, TR-associated proteins; TRE, thyroid hormone response element; TSA, trichostatin A.

SRCs interact directly with liganded NRs and serve as adapter molecules to recruit other coactivators such as p300/CBP. Furthermore, some investigators have found certain SRCs (*i.e.* SRC-1 and SRC-3) to possess a weak intrinsic acetyltransferase (AT) activity (10, 11), although others have been unable to detect this activity (12, 13). p300/CBP and PCAF are potent acetyl transferases that can acetylate histones and a variety of transcription-related factors (14, 15), as well as interact with components of the basal transcriptional machinery (16, 17). p300/CBP is recruited to liganded NRs indirectly via the SRCs (5, 7), whereas PCAF is part of a multipolypeptide complex that can interact directly with liganded NRs and p300/CBP (18, 19). The TRAP complex is a multipolypeptide coactivator complex that interacts with liganded NRs via the TRAP220 subunit and may play a role in recruiting RNA polymerase II to the promoter (8, 20, 21). Recent chromatin immunoprecipitation (ChIP) experiments have demonstrated that after the binding of ligand, TRβ first recruits SRC proteins and p300, resulting in histone acetylation, followed by the TRAP complex (22). Thus, coactivators facilitate transcriptional activation through at least two distinct, but not mutually exclusive, mechanisms: 1) acetylation of histones to facilitate the relief of chromatin-mediated repression; and 2) recruitment of the basal transcriptional machinery.

The packaging of DNA into chromatin represses gene expression (23) and specific biochemical mechanisms have been shown to relieve this repression (24). These include 1) acetylation of the positively charged amino-terminal tails of core histones, which is thought to loosen nucleosome structure and/or disrupt the formation of higher order chromatin structures (25, 26), as well as create new factor binding sites on the histone tails (27); and 2) ATP-dependent chromatin remodeling by complexes such as SWI/SNF, which use the energy of ATP hydrolysis to alter nucleosome structure and/or facilitate nucleosome mobility (24).

The role of chromatin remodeling in TR-mediated transcription has been well established (28). For example, previous studies have provided strong evidence that transactivation by liganded TRβ/RXRα heterodimers appears to involve both HATs and ATP-dependent chromatin remodeling complex(es) (29, 30). However, additional studies have shown that histone acetylation itself is insufficient to fully activate the T_3-regulated TSHα promoter (31). Thus, both chromatin remodeling and covalent histone modifications are important for the function of transcriptional activators such as liganded TRs.

Although it is clear that transcriptional activation by TRs involves a number of distinct coactivators, how those coactivators function together to facilitate TR-dependent transcription is not clear. Both the SRC coactivators and TRAP complex interact directly with liganded TRs (22, 32). It is not yet clear whether the SRC proteins and TRAP complex represent two distinct activation pathways or if they work in a sequential manner to facilitate activation by NRs (20). However, the SRC proteins and p300/CBP appear to function together to form an activation pathway. p300 is indirectly recruited to liganded TRβ during the activation process through interactions with SRC proteins, as illustrated by the fact that p300 does not exhibit strong direct binding to the receptor (33) and that deletion of the SRC interaction domain in p300 greatly diminishes the coactivator activity of p300 with both TRβ and estrogen receptor (ER; Refs. 33 and 34).

A biochemically defined *in vitro* chromatin-based transcription system is useful for addressing a number of questions related to the molecular mechanisms of transcriptional regulation. We have now established a TRβ-dependent, T_3-responsive *in vitro* transcription system using chromatin templates, HeLa cell nuclear extract, and purified recombinant receptor proteins and coactivator proteins. Using this system, we show that TRβ/RXRα heterodimers induce promoter-proximal disruption of nucleosomal arrays upon binding to chromatin and that acetylation of nucleosomal histones enhances T_3-dependent activation. We also demonstrate that both recombinant SRC and p300 proteins facilitate T_3-dependent activation and that the primary role of SRC coactivators is to recruit p300. Finally, we show that multiple functional domains in p300 are critically important for its coactivator activity with TRβ. Together, our results demonstrate how distinct coactivators function with liganded TRβ to overcome chromatin-mediated transcriptional repression.

RESULTS

T_3-Independent Induction of Chromatin Remodeling by TRβ/RXRα Heterodimers *in Vitro*

Recombinant *Xenopus* and human TRβ and RXRα proteins (xTRβ, xRXRα, hTRβ, and hRXRα), including a transcriptionally inactive xTRβ mutant (TRβm) containing a deletion of the last nine amino acids of the AF-2 activation domain, were purified to at least 85% purity in all cases and to near homogeneity in most cases (Fig. 1A and data not shown). In the functional studies described herein, no significant differences between the *Xenopus* and human receptor proteins were observed. Thus, we show only one example for each experiment and refer to the receptors collectively as TRβ and RXRα for simplicity. Gel mobility shift assays (Fig. 1B) revealed that both TRβ and TRβm bound efficiently to a consensus DR4 TRE (5′-AGGTCAnnnnAGGTCA-3′) as TRβ/RXRα heterodimers. Thus, the purified recombinant receptors are competent for DNA binding. The DR4 TRE was selected for the experiments described herein due to its selectivity for TRβ/RXRα heterodimers over TRβ homodimers (*i.e.* TRβ/RXRα heterodimers bind to the DR4 TRE approximately 20-fold more efficiently than TRβ homodimers; Refs. 35–37).

To characterize further the recombinant receptors, we tested whether TRβ/RXRα could bind to TREs in chromatin by deoxyribonuclease (DNase) I footprinting

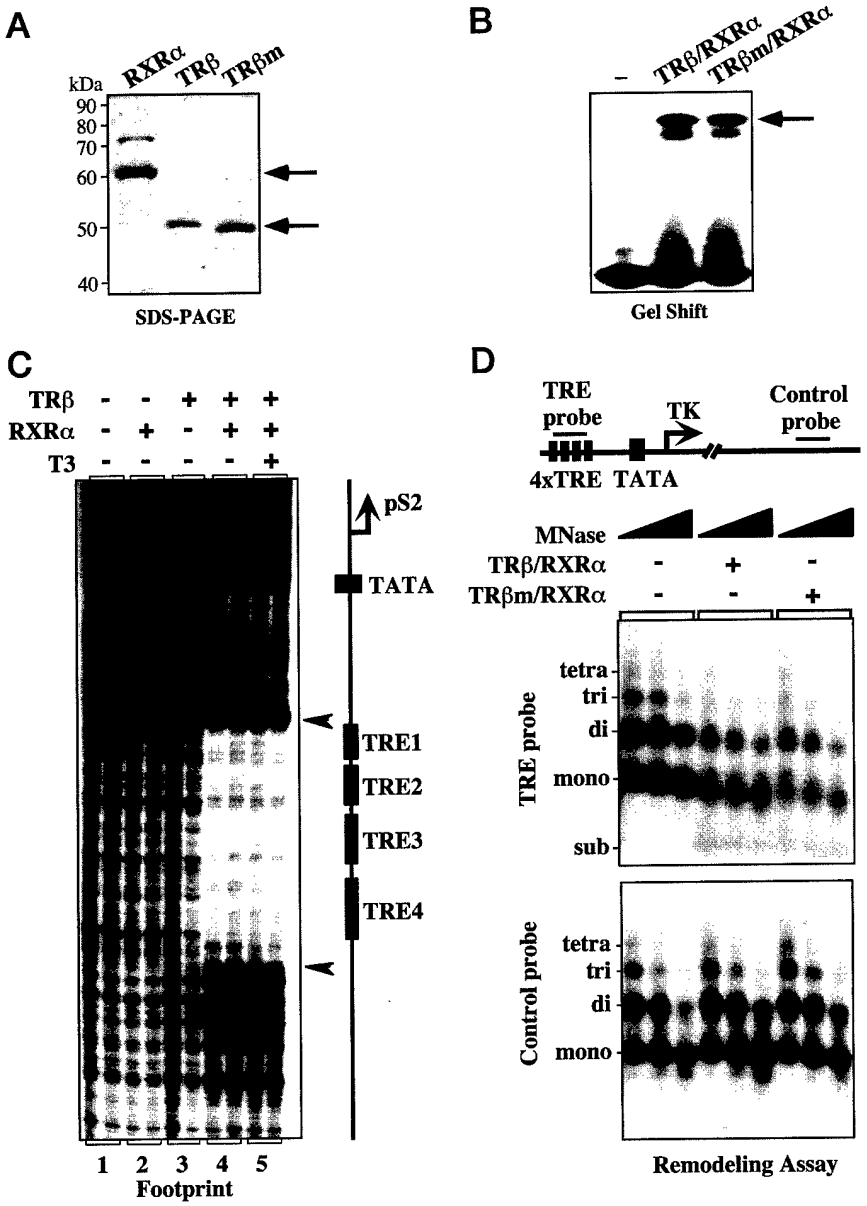

Fig. 1. Recombinant TRβ/RXRα Heterodimers Bind to Chromatin and Induce Chromatin Remodeling

A, Purified recombinant RXRα, TRβ, and TRβmut (TRβm) proteins were analyzed by SDS-PAGE with staining using Coomassie Brilliant Blue R-250. The purified receptors are marked by *arrows*, and the positions of molecular weight markers are indicated. B, Purified recombinant TRβ and TRβm proteins bind to a TRE (DR4) element primarily as heterodimers with RXRα (receptor-DNA shifted complexes are indicated by an *arrow*). The purified TRβ proteins were mixed in 1:1 molar ratio with RXRα proteins and used for gel shift analysis. C, T_3-independent binding of TRβ/RXRα to chromatin assembled *in vitro*. 4xTRE-pS2 (100 ng) was assembled into chromatin with or without receptors (15 nM each) as indicated. The DNase I-primer extension footprinting experiments were carried out in duplicate. A schematic diagram of the 4xTRE-pS2 template is shown *(side)* and the positions of the TREs are indicated. The major hypersensitive sites flanking the TREs are indicated by the *black arrows*. Note that no significant protection is observed in lanes with TRβ or RXRα alone. D, TRβ/RXRα heterodimers induce localized, T_3- and AF-2-independent chromatin remodeling as revealed by an MNase array disruption assay using the template 4xTRE-TK assembled into chromatin *in vitro*. A schematic diagram of the 4xTRE-TK template is shown *(top)*. The positions of the subnucleosomal (sub), mono-, di-, tri-, and tetranucleosomal fragments are indicated. The filter was hybridized with a TRE probe (*top panel*), stripped, and then rehybridized with a control probe (*bottom panel*).

(Fig. 1C). Using the 4xTRE-pS2 template assembled into chromatin *in vitro*, we assessed the binding of RXRα (lane 2), TRβ (lane 3), or TRβ/RXRα heterodimers (lanes 4 and 5). Each binding reaction was partially digested with DNase I and the resulting DNA products were analyzed by primer extension. Addition

of TRβ/RXRα heterodimers resulted in the protection of all four TRE sites in both the absence (lane 4) and presence of T_3 (lane 5), consistent with previous studies showing that TRβ/RXRα can bind to TREs in chromatin and repress transcription in a hormone-independent manner (29). These results also illustrate the importance of heterodimerization with RXRα for efficient binding of TRβ to the DR4 TRE in chromatin (compare lanes 3 and 4).

Next, we performed micrococcal nuclease (MNase) array disruption assays to determine if TRβ/RXRα heterodimers could induce chromatin remodeling. Hybridization using a TRE-specific probe revealed that the addition of TRβ/RXRα heterodimers in the absence of T_3 led to chromatin remodeling, evidenced by a substantial loss of a defined nucleosomal ladder (Fig. 1D, TRE probe). Importantly, chromatin remodeling was localized to the promoter-proximal region, as shown in subsequent experiments with the same blots using a control probe that hybridizes about 2 kb downstream of the TRE (Fig. 1C, control probe; note the intact MNase ladder). Subnucleosomal DNA fragments, representing nucleosome-free DNA fragments protected by the binding of TRβ/RXRα heterodimers, were detected in all samples containing receptors (Fig. 1D, TRE probe, "Sub") but were not detected using the control probe. Heterodimers containing the transcriptionally inactive TRβm also induced efficient chromatin remodeling (Fig. 1D), demonstrating that the AF-2 domain is dispensable for the chromatin remodeling activity. Subsequent experiments comparing chromatin remodeling induced by TRβ/RXRα heterodimers in the presence or absence of ligand indicated that addition of T_3 had no effect on the extent of chromatin remodeling (data not shown). In addition, chromatin remodeling was observed regardless of whether the TRβ/RXRα heterodimers were added during or after chromatin assembly (data not shown). Together, the footprinting and nucleosome disruption assays indicate that the binding of TRβ/RXRα heterodimers to chromatin induces promoter-proximal chromatin remodeling, even with transcriptionally inactive receptors (*i.e.* unliganded or AF-2 mutant).

Transcriptional Repression and Activation by TRβ/RXRα Heterodimers *in Vitro*

The addition of either TRβ/RXRα or TRβm/RXRα heterodimers to unassembled ("naked") 4xTRE-TK DNA template led to a strong repression of transcription (Fig. 2A, lanes 1–5). This repression required the presence of TREs, as a control template lacking TREs was

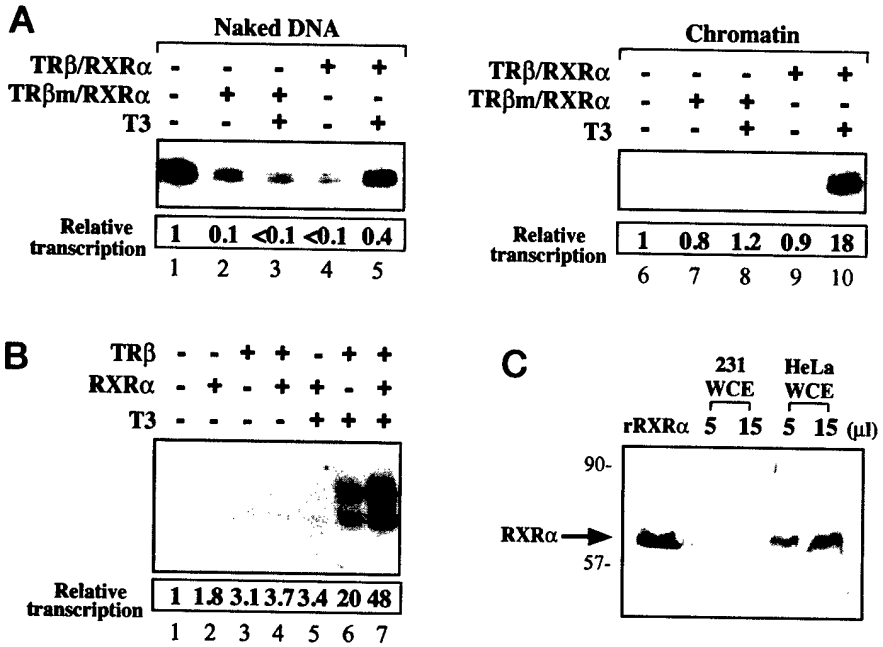

Fig. 2. Transcriptional Activation by TRβ/RXRα with Naked DNA and Chromatin Templates

A, Comparison of transcriptional activation by TRβ/RXRα on naked DNA *(left panel)* and chromatin templates *(right panel)* using *in vitro* transcription assays with the 4xTRE-TK template. In lanes where the addition of T_3 is indicated, TRβ/RXRα or TRβm/RXRα heterodimers (15 nM) were preincubated with 1 μM T_3 for 20 min before being added to the transcription reactions. The transcripts were detected by primer extension. The level of transcription in the absence of ligand and receptors (lanes 1 and 6) was arbitrarily designated as 1. B, The addition of both TRβ and RXRα is required for efficient ligand-dependent transcription with chromatin templates *in vitro*. *In vitro* chromatin assembly and transcription experiments were performed as described in panel A using the 4xTRE-E4 template. TRβ, RXRα, and were added as indicated. C, Western blot of HeLa cell nuclear extract demonstrating the presence of RXRα. Two different volumes of the extract were analyzed as indicated. Aliquots of recombinant RXRα and MDA-MB-231 breast cancer cell nuclear extract were run in adjacent lanes for comparison.

only marginally affected (data not shown). Although preincubation of the receptors with T_3 relieved repression by unliganded TRβ/RXRα (compare lanes 4 with 5), no activation above the basal level was observed (compare lanes 1 with 5). In contrast, preincubation with T_3 failed to relieve repression by TRβm/RXRα (lane 3). Thus, an intact AF-2 activation domain is not required for basal repression by unliganded TRβ but is required for the relief of repression in the presence of T_3.

Several important differences were observed when the template was assembled into chromatin and used for *in vitro* transcription with TRβ/RXRα. First, as expected, basal levels of transcription were dramatically reduced when compared with the naked DNA template (about 50- to 100-fold; Fig. 2A, compare lanes 1 and 6). Furthermore, a strong T_3-dependent activation was observed (Fig. 2A, compare lanes 6 and 10). This activation required an intact AF-2 activation domain because TRβm/RXRα failed to activate under the same conditions (Fig. 2A, lanes 6–8). Thus, this *in vitro* system accurately recapitulates T_3-dependent activation and the requirement for an intact AF-2 domain. T_3-dependent activation with chromatin templates is not specific to the thymidine kinase (TK) promoter as both the human pS2 and adenovirus E4 promoters gave similar results (see below). Interestingly, repression by unliganded TRβ/RXRα was not observed with the chromatin templates, most likely due to the fact that chromatin assembly itself repressed transcription to a marginally detectable level.

Significant contributions of TRβ homodimers to the receptor-dependent transcriptional effects shown in Fig. 2A are unlikely for the following reasons. First, as mentioned above, TRβ homodimers do not bind strongly to the DR4 TRE in our reporter templates (35–37). Second, RXRα is required for efficient binding of TRβ to the DR4 TRE-containing chromatin templates used in our *in vitro* transcription studies, as shown in Fig. 1C. To examine this issue more directly, we performed *in vitro* transcription experiments with chromatin templates using TRβ in the presence or absence of RXRα (Fig. 2B). As noted above, the addition of recombinant TRβ/RXRα heterodimers gave a robust T_3-dependent transcriptional response in this assay (lane 7). The addition of TRβ without RXRα also gave a T_3-dependent transcriptional response, but the magnitude of the effect was about 2.5-fold less than that observed with TRβ/RXRα heterodimers (lane 6). Why would the addition of TRβ alone give a transcriptional response when TRβ alone does not bind to DR4 TRE with chromatin templates? This is likely due to the presence of RXRα (and possibly other RXR subtypes) in the HeLa cell nuclear extracts that we used for the *in vitro* transcription studies (see the Western blot in Fig. 2C). Because the footprinting assays were performed in the absence of the HeLa cell nuclear extract, the effects of endogenous HeLa cell RXRα were not observed. Taken together, the available data indicate that the transcriptional responses that we observed are due to TRβ/RXRα heterodimers and not TRβ homodimers.

Histone Acetylation Enhances TRβ-Mediated Transcription

The levels of histone acetylation *in vivo* are ultimately determined by the balance of HAT and histone deacetylase (HDAC) activities. Our *in vitro* chromatin assembly and transcription system contains both endogenous HATs and HDACs, as well as acetyl-coenzyme A (CoA; Kraus, W. L., unpublished observations). To test the effect of histone acetylation on T_3-dependent activation *in vitro*, we used a specific HDAC inhibitor, trichostatin A (TSA), to block deacetylation and increase the overall levels of histone acetylation. As shown in Fig. 3A, the addition of TSA (1 μM) to the chromatin assembly reaction led to an approximately 2-fold increase in bulk histone H4 acetylation (compare lanes 1 and 4), whereas addition of liganded or unliganded TRβ/RXRα alone had no effect (lanes 2 and 3). *In vitro* transcription experiments demonstrated that the addition of TSA enhanced T_3-dependent activation by about 2.5-fold (Fig. 3B, compare lanes 4 and 8). The addition of TSA also enhanced transcription in the absence of receptors (lanes 1, 2, 5, and 6), indicating that increased acetylation of bulk histones has a general stimulatory effect on both basal and TRβ-mediated transcription.

To assess whether liganded TRβ/RXRα is able to induce localized chromatin acetylation through the recruitment of coactivators with intrinsic HAT activities, we used an *in vitro* ChIP assay (Fig. 3C). 4xTRE-TK was assembled into chromatin in the presence or absence of receptors, ligand, and TSA, as indicated. The chromatin templates were then used in reactions under transcription conditions, except that ribonucleotide 5′-triphosphates were omitted to avoid complications due to potential effects of transcriptional elongation on acetylation. After extensive MNase digestion, the chromatin was immunoprecipitated with antibodies specific for the acetylated forms of either histone H4 or H3, and the coimmunoprecipitated DNA was analyzed by slot-blot hybridization. The presence of liganded TRβ/RXRα led to about a 3-fold enhancement of H4 and H3 acetylation in the promoter region, as evidenced by an enrichment of DNA in lanes 8 and 12 compared with lanes 5 and 9, respectively, with a probe specific for the TATA box (*top panel*). This ligand- and receptor-dependent enhancement of H4 and H3 acetylation was localized to the promoter region, as a control probe located about 2 kb downstream of the promoter showed little, if any, increase above the basal level (*bottom panel*). In contrast, TSA treatment led to about a 7.5-fold increase in acetylation in both the proximal and distal locations (lanes 6 and 10), indicating that the hyperacetylation of chro-

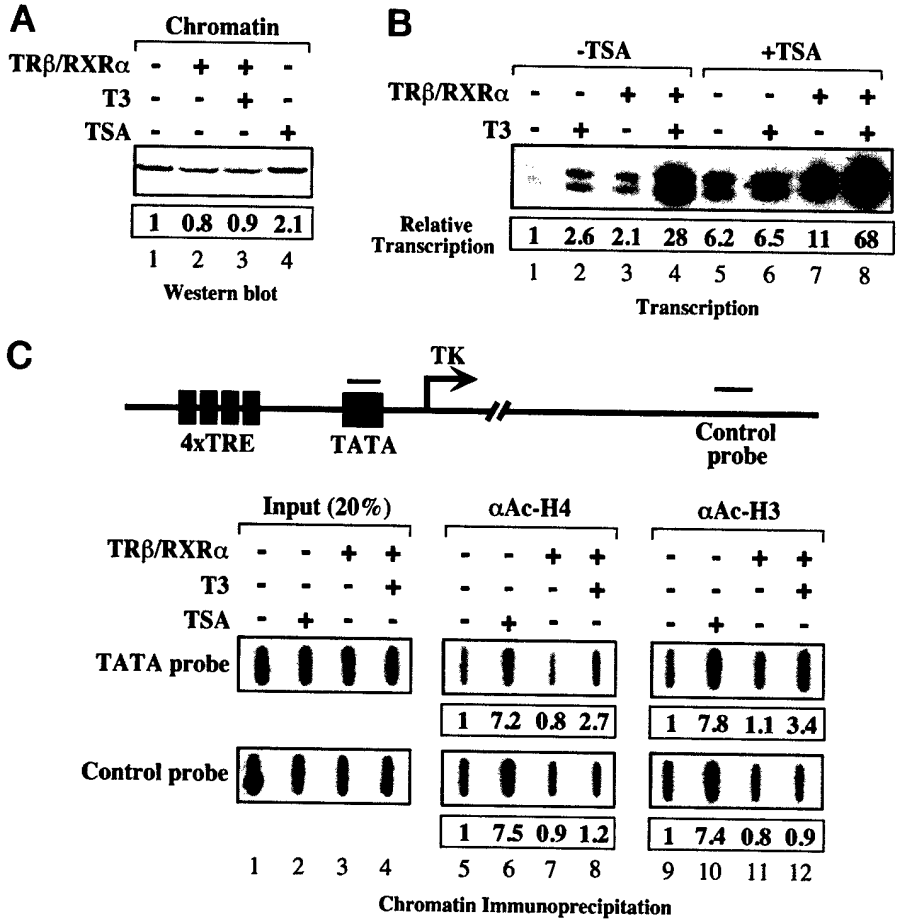

Fig. 3. Promoter-Targeted Histone Acetylation Enhances Transcriptional Activation by Liganded TRβ/RXRα

A, TSA treatment enhances the level of histone acetylation in chromatin. Reactions were identical to the transcription assays described in Fig. 2B except that 1 μM of TSA was added to the reaction in lane 4. Histone acetylation was analyzed by Western blotting using an anti-acetylated H4 antibody. B, TSA treatment enhances T_3-dependent activation. Chromatin templates were assembled with or without TSA (1 μM) as in (A) and used for transcription assays. The relative transcription was quantified using a PhosphorImager and represents the average results from two independent experiments. C, Liganded TRβ/RXRα induces a localized acetylation of histones H4 and H3 as assessed by *in vitro* ChIP assays. A schematic diagram of the 4xTRE-TK construct and the locations of the oligonucleotide probes used for slot blot hybridization are shown *(top)*. Chromatin assembly and incubation with TRβ/RXRα and HeLa cell nuclear extracts were as described in (A). ChIP assays were performed with anti-acetylated H4 and antiacetylated H3 antibodies with subsequent analysis by sequential slot-blot hybridization using a promoter region probe (TATA) and a control probe, as indicated. The data shown are averaged from three independent experiments are standardized to the input signal for each experiment (see representative input at *right*).

matin induced by TSA is not a targeted event. Thus, liganded TRβ/RXRα can target histone acetylation to T_3-activated promoters, presumably through hormone-dependent recruitment of HATs such as p300/CBP and PCAF.

SRC Proteins and p300 Synergistically Stimulate TRβ-Dependent Transcription *in Vitro*

Next, we examined whether the addition of purified recombinant SRC-3 and p300 could stimulate TRβ-dependent activation *in vitro*. Full-length FLAG-tagged SRC-3 protein was purified from microinjected *Xenopus* oocytes (Fig. 4A) and full-length his_6-tagged p300 protein was purified from baculovirus-infected Sf9 cells (Fig. 4C). As shown in Fig. 4B, the addition of increasing amounts of SRC-3 stimulated TRβ-dependent activation approximately 3.5-fold in the presence of T_3. Similar results were also obtained when purified recombinant SRC-1 and SRC-2 proteins were used under similar conditions (data not shown). As shown in Fig. 4D, the addition of p300 stimulated TRβ-dependent activation approximately 4-fold in the presence of T_3. Thus, both SRC proteins and p300 are able to stimulate TRβ-dependent activation with chromatin templates *in vitro*.

To further elucidate the molecular mechanisms by which SRC and p300 stimulate TRβ-dependent activation, we investigated potential synergistic interactions between the two coactivators. Recombinant full-

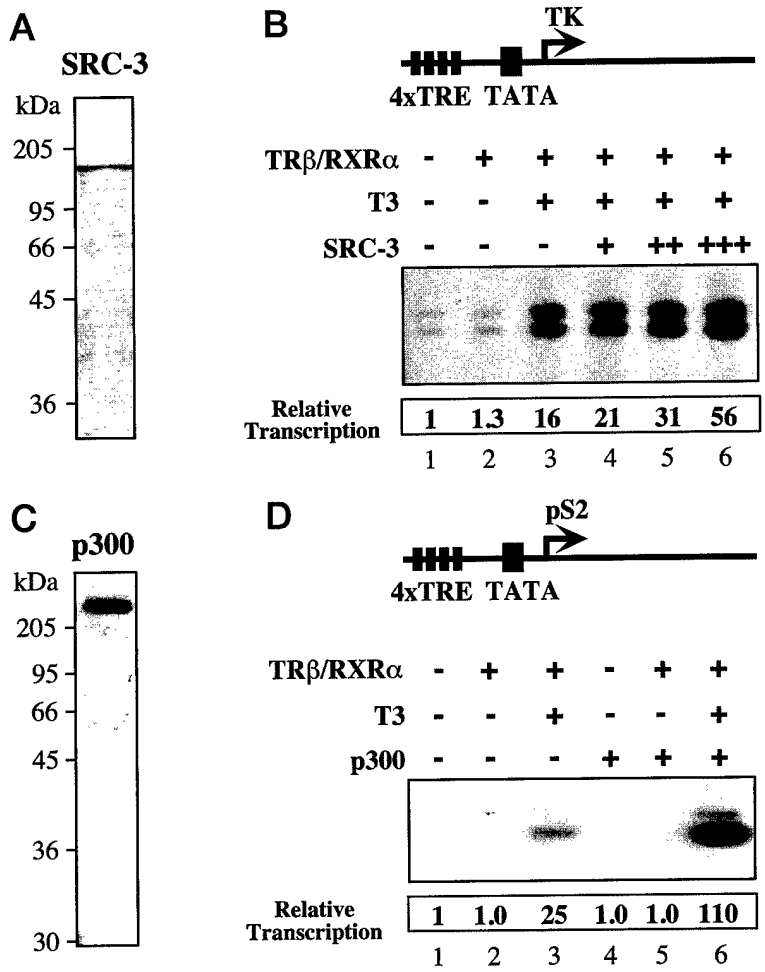

Fig. 4. Recombinant SRC-3 and p300 Enhance TRβ-Dependent Transcription with Chromatin Templates *in Vitro*

A, Purified SRC-3 was analyzed by SDS-PAGE with staining using Coomassie Brilliant Blue R-250. B, Addition of SRC-3 stimulates T_3-dependent transcription with chromatin templates. 4xTRE-TK (see schematic, *top*) was assembled into chromatin and used for *in vitro* transcription in the presence of TRβ/RXRα, T_3, and increasing amounts of SRC-3 protein (1.25 nM, 2.5 nM, and 5 nM), which was added to the reactions in lanes 4 through 6 after chromatin assembly was complete. C, Purified p300 was analyzed by SDS-PAGE with staining using Coomassie Brilliant Blue R-250. D, Addition of p300 stimulates T_3-dependent transcription with chromatin templates. 4xTRE-pS2 (see schematic, *top*) was assembled into chromatin and used for *in vitro* transcription in the presence of TRβ/RXRα, T_3, and p300 protein (5 nM), which was added to the reactions in lanes 4–6 after chromatin assembly was complete.

length SRC-3 protein and an SRC-2 protein containing only the receptor interaction domain (RID) and p300/CBP-interaction domain (PID; amino acids 624-1130; Figs. 5, A and B) were added to *in vitro* transcription experiments with TRβ/RXRα and p300. Note that the amount of p300 was reduced relative to the experiments in Fig. 4 to expose potential synergism with the SRCs. With the E4 promoter, p300 and the SRCs individually stimulated TRβ-dependent transcription about 10-fold and 2- to 3-fold, respectively (Fig. 5C, *top*; lanes 2–5). When added together, p300 and the SRCs acted synergistically, producing about a 20-fold increase in TRβ-dependent transcription relative to the same conditions without exogenously-added coactivators [Fig. 5C, *top*; compare lanes 3, 5, and 7 for p300 and SRC-3; lanes 3, 4, and 6 for p300 and SRC-2(RID/PID)]. With the pS2 promoter, p300 stimulated TRβ-dependent transcription about 2-fold, but the SRCs had little or no effect when added alone (Fig. 5C, *bottom*; lanes 2–5). However, when added together, p300 and SRC gave about a 3-fold increase in TRβ-dependent transcription with the pS2 promoter [Fig. 5C, *bottom*; see lanes 3, 5, and 7 for p300 and SRC-3; lanes 3, 4, and 6 for p300 and SRC-2(RID/PID)]. Interestingly, the effects of SRC-2(RID/PID) were generally similar to the effects observed with full-length SRC-3 (Fig. 5C, compare lanes 4 and 5, and 6 and 7), indicating that the receptor and p300/CBP interaction domains of SRC-2 are sufficient to mediate coactivator activity. Together, our results indicate that p300 and SRC proteins function synergistically to stimulate TRβ-mediated transcription with chromatin templates,

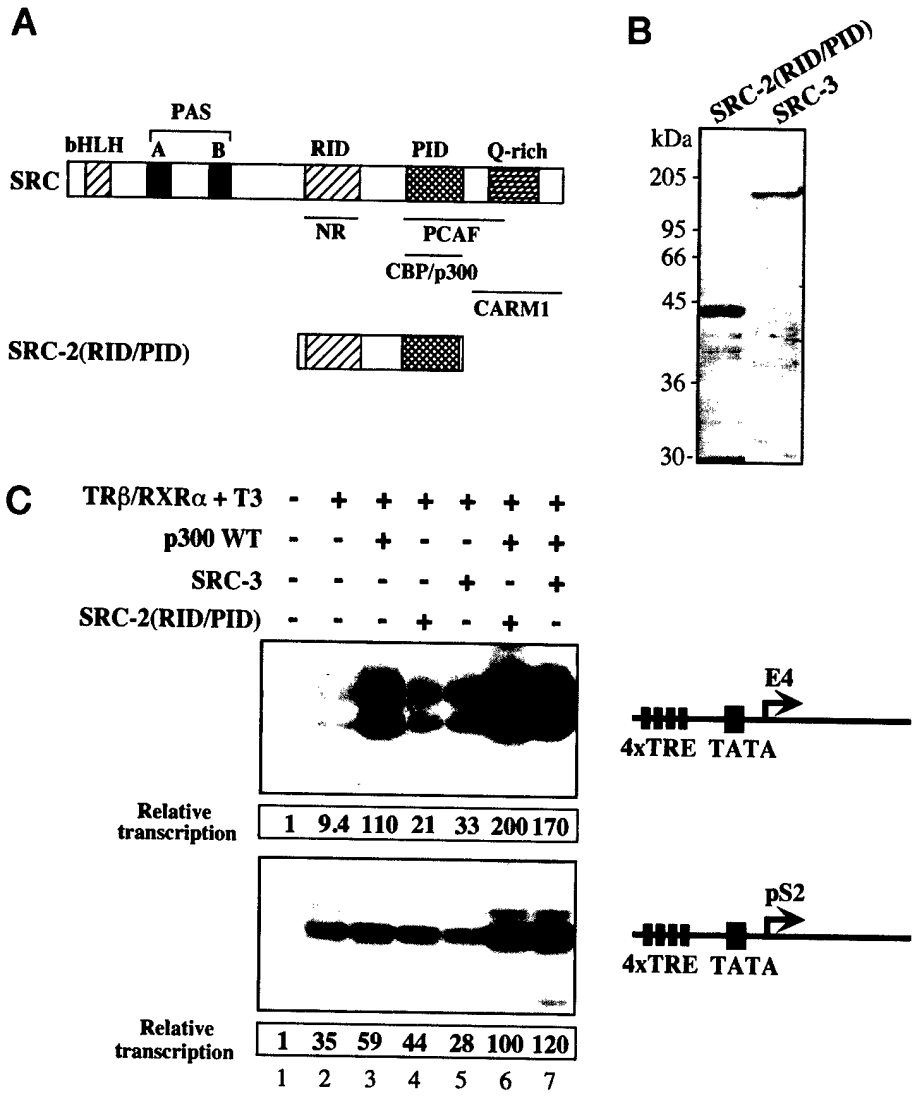

Fig. 5. SRC and p300 Synergistically Stimulate Ligand-Dependent Transcription by TRβ/RXRα

A, Generalized schematic diagram of the SRC coactivators, including the following functional domains: basic helix-loop-helix (bHLH) domain, Per/Arnt/Sim (PAS) domain, RID, PID, and the glutamine (Q)-rich region. Also indicated are regions that bind PCAF and CARM1. SRC-2(RID/PID) contains only amino acids 624-1130 of SRC-2. B, Purified SRC-2(RID/PID) and SRC-3 were analyzed by SDS-PAGE with staining using Coomassie Brilliant Blue R-250. C, Effects of the addition of SRC and p300 on TRβ-mediated transcription with chromatin templates. 4xTRE-E4 and 4xTRE-pS2 (see schematics at *right*) were assembled into chromatin and used for *in vitro* transcription in the presence of TRβ/RXRα (4.5 nM), T_3 (1 μM), p300 (0.5 nM), SRC-3 (5 nM), and SRC-2(RID/PID) (5 nM), as indicated. Note that the p300 amount was reduced compared with the experiments in Fig. 4 to show synergism with the SRCs. The RNA products were analyzed by primer extension.

and that a central role for SRC is to recruit p300 to the liganded receptor.

Multiple Domains of p300 Are Required for Its Coactivator Activity with TRβ

p300/CBP contains multiple functional domains, including a bromodomain, three cys/his (CH)-rich regions, an acetyltransferase domain, and an SRC interaction domain (Fig. 6A). The bromodomain is found in many chromatin- and transcription-related factors and is believed to be important in histone binding and other protein-protein interactions (38–40). The CH3 region is a protein interaction domain that interacts with a variety of factors, including PCAF (15), transcription factor IIB (TFIIB) (41), and RNA polymerase II (42). To further explore the role of p300 in TRβ-mediated transcription, we used a set of previously characterized p300 mutants (Fig. 6A; Ref. 34). The mutants included: 1) a bromodomain deletion (ΔBromo), 2) an AT mutant (MutAT2), 3) a CH3 region deletion (ΔCH3), and 4) an SRC interaction domain deletion (ΔSRC). The purified mutant p300 proteins (Fig. 6B) were used in TRβ-dependent transcription reactions with the

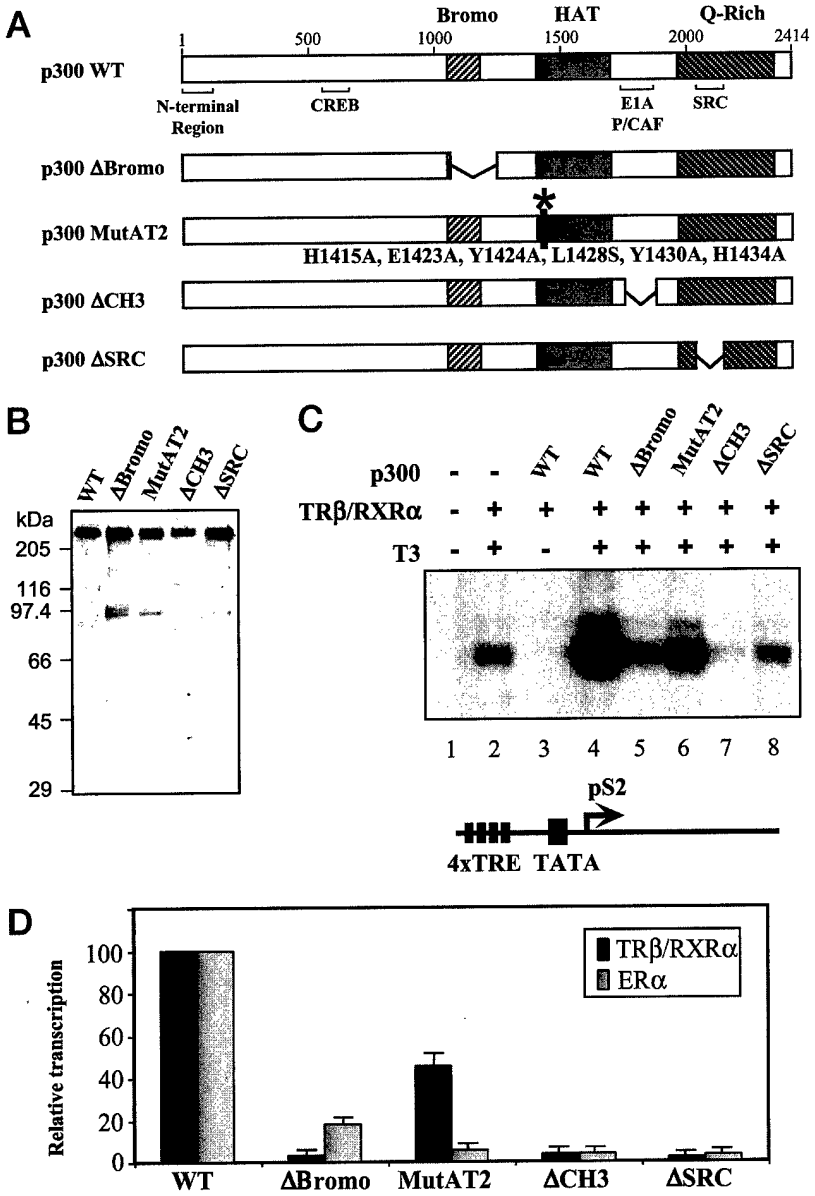

Fig. 6. Multiple Functional Domains of p300 Are Important for Stimulating TRβ-Dependent Transcription with Chromatin Templates

A, Schematic diagrams of wild-type and mutant p300 proteins used in this study showing the various functional domains of p300: bromodomain (Bromo), AT domain, CH-rich region 3 (CH3), and the glutamine (Q)-rich region. Also indicated are regions that bind the adenovirus E1A protein, PCAF, TFIIB, and RNA polymerase II (pol II). B, Purified wild-type and mutant p300 proteins were analyzed by SDS-PAGE with staining using Coomassie Brilliant Blue R-250. C, Effects of the addition of wild-type and mutant p300 proteins (5 nM) on TRβ-mediated transcription with chromatin templates. 4xTRE-pS2 (see schematic at *bottom*) was assembled into chromatin and used for *in vitro* transcription as described in Fig. 4C. D, Graphical representation of the results in (C) shown in comparison with the results from similar experiments performed with ERα. Only the contributions from the exogenously added recombinant p300 proteins are included in the results. Each *bar* represents the mean + the SEM from three independent experiments.

4xTRE-pS2 template assembled into chromatin. The use of these mutants, in conjunction with the additional experimental approaches shown in Figs. 7 and 8, allowed us to address the role of p300-SRC interactions and p300 HAT activity in TRβ-mediated transcription, as well as questions about the other functional domains in p300.

Deletion of the SRC interaction domain dramatically reduced the ability of p300 to enhance TRβ-dependent transcription (Fig. 6C, compare lanes 4 and 8). This result further supports the conclusion that recruitment of p300 to TRβ via the SRCs is essential for p300 coactivator activity with TRβ. Deletion of either the bromodomain or the CH3 region also led to a substan-

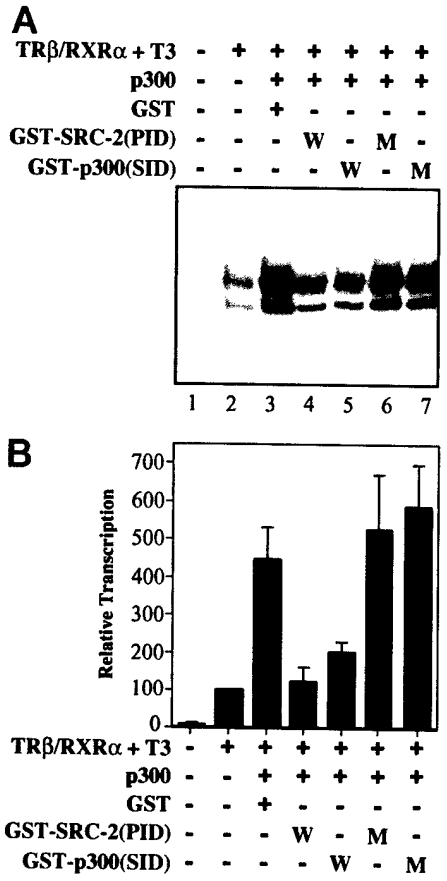

Fig. 7. p300-SRC Interactions Are Required for Full Transcriptional Activation by TRβ

A, Polypeptide inhibitors reduce p300 stimulated TRβ-activated transcription. The template 4xTRE-E4 (see schematic Fig. 5C) was assembled into chromatin and used for *in vitro* transcription in the presence of TRβ/RXRα (4.5 nM), T_3 (1 μM), and p300 (5 nM) and GST peptide (225 nM) where indicated. Wild-type (W) or mutant (M) versions of GST-SRC2(PID) and GST-p300(SID) (225 nM) were added as indicated before transcription. B, Graphical representation of results shown in (A). Each *bar* represents the mean + the SEM from four experiments with the level of transcription in the presence of liganded receptor set at 100%.

tial reduction in p300 activity (Fig. 6C, compare lanes 4, 5, and 7), indicating that both domains are important for p300 coactivator activity with TRβ. The p300 MutAT2 protein, which has about 1% of wild-type HAT activity (34), showed a 50% reduction in coactivator activity with TRβ. Thus, p300 HAT activity is also needed for full coactivator activity with TRβ (see additional experiments presented below that address this issue further). For comparison, the results from multiple experiments with TRβ were quantified and plotted *vs.* results from similar experiments using ERα (Fig. 6D). It is clear from this comparison that multiple p300 functional domains are required for maximal coactivator activity with both TRβ and ERα with chromatin templates.

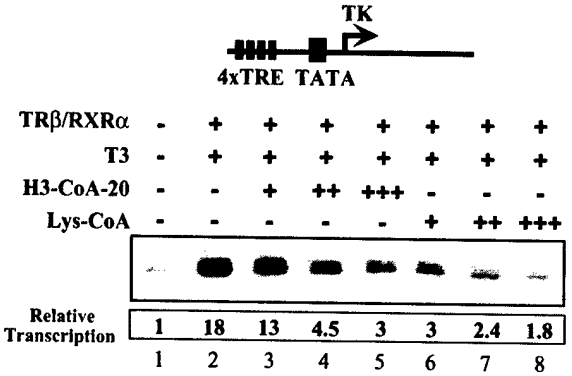

Fig. 8. Both p300 and PCAF HAT Activities Contribute to TRβ-Mediated Transcription with Chromatin Templates

The 4xTRE-TK reporter (see schematic, *top*) was assembled into chromatin and used for *in vitro* transcription in the presence of TRβ/RXRα, T_3, and increasing amounts of either H3-CoA-20 (a PCAF-selective HAT inhibitor) or Lys-CoA (a p300/CBP-selective HAT inhibitor) as indicated (+, 2 μM; ++, 4 μM; +++, 8 μM). The transcription and primer extension assays were essentially as described in Fig. 1 except that HeLa nuclear extracts were preincubated with the indicated amounts of the HAT inhibitors for 10 min on ice before addition to the transcription assays. The level of transcription in the absence of ligand and receptors (lane 1) was designated as 1.

Inhibition of p300-SRC Interactions or p300 HAT Activity Inhibits TRβ-Mediated Transcription

To evaluate further the role of p300-SRC interactions and p300 HAT activity in TRβ-mediated transcription, we used previously characterized polypeptide inhibitors of p300-SRC interactions and chemical inhibitors of HAT activity in chromatin transcription experiments with TRβ. First, to block p300-SRC interactions, we used a set of glutathione-S-transferase (GST)-fused polypeptides containing either the PID of SRC-2 or the SRC interaction domain (SID) of p300. These polypeptides, which have previously been shown to be potent inhibitors of ERα-mediated transcription (43), specifically and competitively block interactions between the endogenous SRC proteins in the HeLa cell transcription extract and exogenously-added p300 (43). Both GST-SRC-2(PID) and GST-p300(SID) inhibited the enhancement of TRβ-mediated transcription by p300 (Fig. 7A, compare lanes 2 and 3 with lanes 4 and 5; Fig. 7B). The inhibitory effects were not observed with mutant versions of the same polypeptides ("Mut") that fail to bind their cognate partners (Fig. 7A, compare lanes 4 and 5 with lanes 6 and 7; Fig. 7B). These results complement our results with the p300ΔSRC mutant protein (Fig. 6C) and illustrate further the critical role that p300-SRC interactions play in TRβ-mediated transcription with chromatin templates.

Second, to explore further the role of p300 HAT activity in TRβ-mediated transcription, we used Lys-CoA, a chemical inhibitor that selectively blocks p300/CBP HAT activity (44–46). Lys-CoA is at least 200-fold

more potent at blocking p300/CBP HAT activity than the HAT activities of GCN5, PCAF, Mof, and Esa1 (Cole, P. A., unpublished observations). For comparison, we also used H3-CoA-20, a chemical inhibitor that selectively blocks PCAF HAT activity (44). As shown in Fig. 8, increasing amounts of either inhibitor caused a reduction in TRβ-mediated transcription, although the p300/CBP-selective Lys-CoA was a more effective inhibitor than the PCAF-selective H3-CoA-20. These results suggest a role for both p300/CBP and PCAF HAT activities in TRβ transcriptional activity, although nonspecific effects of these inhibitors cannot be ruled out. The results with Lys-CoA are in agreement with our results using the p300 HAT mutant (Fig. 6), further supporting a role for p300 HAT activity in TRβ-mediated transcription. Differences in the magnitude of the effects with the chemical inhibitor and the p300 mutant may be due to promoter-specific effects (pS2 *vs.* TK) or the fact that the Lys-CoA targets the endogenous p300 in the transcription extract, whereas the p300 HAT mutant is an addition to the endogenous levels of p300. Nonetheless, both approaches indicate an important role for p300 HAT activity in TRβ-mediated transcription. Furthermore, the possible contribution of PCAF HAT activity to TRβ-mediated transcription may also provide an explanation for the lack of an absolute requirement for p300 HAT activity under certain promoter contexts (Fig. 6).

DISCUSSION

We have used an *in vitro* chromatin assembly and transcription system that accurately recapitulates ligand- and AF-2-dependent transcriptional activation by TRβ/RXRα heterodimers to study TRβ-mediated transcription with chromatin templates. Together, our results demonstrate that a biochemical approach that includes chromatin is a useful way to study the mechanisms of TRβ-mediated transcription. Below, we highlight the most significant results from our studies.

Chromatin Remodeling by TRβ/RXRα Heterodimers *in Vitro*

In a previous report, we showed that TRβ/RXRα heterodimers induce an extensive, localized disruption of chromatin only in the presence of T_3 in *Xenopus* oocytes (47). In contrast, we show herein that TRβ/RXRα heterodimers induce a localized disruption of chromatin in a T_3-independent manner (Fig. 1D). In fact, other receptors, such as ERα, progesterone receptor, and retinoic acid receptor (RAR)-α/RXRα, also induce ligand-independent remodeling in this *in vitro* system (data not shown; Refs. 48 and 49). The discrepancy between these two systems is most likely due to differences in the concentrations or types of ATP-dependent chromatin remodeling factors present in *Xenopus* oocytes and the *Drosophila* embryo extracts used for our *in vitro* chromatin assembly experiments. *Drosophila* embryo extracts are highly enriched for ATP-dependent chromatin remodeling factors such as nucleosome remodeling factor (NURF), chromatin accessibility complex (CHRAC), and ATP-utilizing chromatin assembly and remodeling factor (ACF) (Refs. 50–52), which are capable of inducing nucleosome sliding without nucleosome displacement (24). The chromatin remodeling induced by TRβ/RXRα in our *in vitro* system (Fig. 1D) most likely reflects the binding of TRβ/RXRα heterodimers to nucleosome-free TREs produced by the actions of chromatin remodeling factors in the absence of specific recruitment. Although TRβ/RXRα-mediated chromatin remodeling *in vitro* is ligand independent, it is likely to be a prerequisite for subsequent transcriptional activation (53). This idea is consistent with a recent report demonstrating that the efficient binding of RARα/RXRα heterodimers to chromatin requires ATP-dependent chromatin remodeling factors (54) and that the ATP-dependent chromatin remodeling factor NURF stimulates transcription activation by the synthetic activator GAL4-VP16 (55).

Role of Histone Acetylation in TRβ-Mediated Transcription

The inclusion of the HDAC inhibitor TSA in the *in vitro* system increased the acetylation of histones over the whole population of nucleosomes on the plasmid templates (Fig. 3C). The increased acetylation was correlated with a 2- to 3-fold increase in ligand-dependent transcription by TRβ/RXRα (Fig. 3B). However, increased acetylation was also correlated with increased transcription in the absence of receptor and by unliganded TRβ/RXRα, consistent with the idea that acetylation of histones has a general positive effect on transcription (Fig. 3B). Thus, although increased histone acetylation is likely to enhance TRβ transcriptional activity, the effects of TSA are not specific. In contrast, TRβ-mediated histone acetylation in our *in vitro* ChIP assay was specific for the promoter region (Fig. 3C). These results are consistent with recent reports that liganded RARα and ERα are able to induce histone acetylation at the promoters of hormone target genes in mammalian cells (56, 57) and in biochemical assays (43, 54). In agreement with recently reported ChIP data (22), our results indicate that liganded TRβ/RXRα, once bound to TRE elements in chromatin, can recruit HATs such as p300/CBP and PCAF, which in turn acetylate the adjacent nucleosomes. The acetylation of nucleosomes in the promoter region helps to relieve chromatin-mediated repression and facilitate transcriptional activation.

With regard to the specific HAT enzymes involved in TRβ-mediated transcription, our results with the p300 HAT mutant and the chemical inhibitor Lys-CoA indicate that p300 HAT activity (and possibly CBP HAT activity, as well) is required for full transcriptional activation by TRβ with chromatin templates (Figs. 6 and

8). These results are in agreement with previous studies using *Xenopus* oocytes in which the HAT activity of p300 was shown to be required for the stimulation of T_3-dependent activation by TRβ/RXRα (33). Our results with the chemical inhibitor H3-CoA-20 (Fig. 8) suggest a role for PCAF HAT activity in TRβ-dependent transcription with chromatin templates as well, although nonspecific effects with the H3-CoA-20 inhibitor cannot be ruled out (Ref. 44; and Cole, P. A., unpublished observations). Interestingly, we found little evidence for a contribution of the putative SRC HAT activity in TRβ-mediated transcription, as a fragment of SRC-2 containing only the receptor and p300/CBP interaction domains gave activity similar to that of full-length SRC proteins (Fig. 5 and data not shown). Together, our results indicate that specific coactivators and their associated enzymatic actions on nucleosomal histones play important roles in transcriptional regulation by TRβ.

A Critical Role for SRC-p300 Interactions in TRβ-Mediated Transcription

Our studies with TRβ indicate that p300 is recruited indirectly to promoter-bound TRβ through its interaction with SRC proteins. This conclusion is supported by the following results. First, we showed that purified recombinant SRC-3 and p300 synergistically enhance TRβ-mediated transcription with chromatin templates (Fig. 5C). Second, a fragment of SRC-2 containing only the receptor and p300/CBP interaction domains was able to synergize with p300 and was functionally equivalent to full-length SRC-3 (Fig. 5C). Third, a p300 mutant lacking the SRC interaction domain (p300ΔSRC) was unable to function as a coactivator for TRβ/RXRα (Fig. 6D). Finally, polypeptide inhibitors that directly interfere with TRβ-SRC-p300 interactions blocked the ability of p300 to stimulate TRβ-mediated transcription. Thus, a primary role for the SRC proteins is to recruit p300/CBP to liganded receptors, and the TRβ-SRC-p300/CBP pathway constitutes one pathway for the activation of T_3-dependent transcription by TRβ. These conclusions apply to other nuclear receptors and are further supported by some recent cell-based studies (12, 22, 33, 58), as well as *in vitro* transcription analyses (43, 48, 59).

Multiple Domains in p300 Contribute to Its Coactivator Activity with TRβ

Both p300 and CBP are multifunctional proteins that stimulate the transcriptional activity of many different transcriptional activators, including NRs (17). Our results indicate that in addition to the SRC-interaction domain discussed above, both the bromodomain and CH3 region are critically important for p300 coactivator activity with TRβ. We have previously shown that both of these domains are critical for p300 coactivator activity with a variety of transcriptional activators, including ERα, nuclear factor-κB p65, and Gal4-VP16 (34). The bromodomain is found in many chromatin- and transcription-related factors and is believed to be important in chromatin binding and/or protein-protein interactions (38–40). We have recently shown that the p300 bromodomain mediates the stable interaction of p300 with chromatin and is important for p300 nucleosomal HAT activity (34, 40). The p300 CH3 region has been found to interact with proteins such as TFIIB and RNA polymerase II (41, 42). Thus, the loss of coactivator activity for p300ΔCH3 could be explained by its inability to interact with or recruit components of the basal transcriptional machinery.

Together, our results suggest a multiple step pathway for transcriptional regulation by liganded TRβ, including chromatin remodeling, recruitment of bridging and HAT coactivators (*e.g.* SRCs and p300/CBP, respectively), targeted histone acetylation, and recruitment of the RNA polymerase II transcriptional machinery. Multiple step pathways may be a universal feature for transcriptional activation by NRs (60). The absence of transcriptional activation above basal levels with naked DNA (Fig. 2A), as well as the requirement for ligand and an intact AF-2 domain (both of which facilitate coactivator recruitment) for activation with chromatin templates (Fig. 2B) suggest that coactivators such as SRC and p300 function by alleviating the repressive effects of chromatin at the promoter. Our results suggest that, for TRβ/RXRα heterodimers, this can be achieved through targeted histone acetylation and specific contacts with the transcriptional machinery.

MATERIALS AND METHODS

Expression and Reporter Plasmids

The bacterial expression constructs for *Xenopus* RXRα (pET15b-xRXRα) and TRβ (pET15b-xTRβ) have been described before (61). The bacterial expression construct for xTRβm with a deletion of the last nine amino acids of the AF-2 domain was generated by replacing the wild-type xTRβ in the pET15b-xTRβ with the xTRβm1 from the pSP64-TRm1 plasmid described previously (47). The 4xTRE-pS2 reporter template is the same as 2xERE-pS2 (62) except that the EREs have been replaced with four TREs. Both the 4xTRE-E4 and 4xTRE-TK reported templates were constructed by inserting four TREs upstream of the adenovirus E4 promoter in pIE0 or the herpes simplex virus TK promoter in pTK-CAT, respectively. The sequence of the DR4 TRE used in our studies is 5′-GATATCAGGTCATTTCAGGTCAGCATGC-3′.

Expression and Purification of Recombinant Proteins

Purification of bacterially expressed his_6-tagged *Xenopus* TRβ, TRβm, and RXRα was by nickel nitrilotriacetic acid (Ni-NTA) agarose chromatography (QIAGEN, Valencia, CA) followed by Mono S chromatography (Amersham Pharmacia Biotech, Arlington Heights, IL). The receptors were eluted from the Mono S column with a salt gradient from 100–500 mM KCl. His_6-tagged human TRβ and RXRα were prepared from baculovirus-infected Sf9 cells by Ni-NTA agarose chromatography as described before for p300 (63). Full-length SRC-3 containing an amino terminal FLAG tag was prepared

from microinjected *Xenopus* oocytes as described (48). Bacterially expressed his-tagged SRC-2(RID/PID; amino acids residues 624-1130) was purified by Ni-NTA agarose chromatography as described before (43). Wild-type and mutant his_6-tagged p300 proteins were prepared from baculovirus-infected Sf9 cells by Ni-NTA agarose chromatography as described before (34, 63). GST-tagged wild-type and mutant polypeptide inhibitors [SRC-2(PID), SRC-2(PID)Mut, p300(SID), p300(SID)Mut] were purified as previously described (43). All purified recombinant proteins were evaluated by SDS-PAGE with staining using Coomassie Brilliant Blue R-250.

In Vitro Chromatin Assembly and Transcription

Chromatin assembly reactions were performed with an S-190 chromatin assembly extract derived from *Drosophila* embryos as previously described (48, 63). TRβ/RXRα or TRβm/RXRα proteins were added to the chromatin assembly reactions either at the beginning of chromatin assembly or after the assembly reactions were complete. SRC and p300 proteins, as well as the polypeptide inhibitors, were added after chromatin assembly, before the addition of transcription extract. *In vitro* transcription reactions were performed with HeLa cell nuclear extracts that were prepared essentially by the method of Dignam *et al.* (64). Transcription reactions were set up under conditions described previously (43, 48, 63). The reactions were performed in duplicate, but single samples from each experiment are shown in the figures. The data were analyzed and quantified with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Each experiment was run a minimum of two separate times, but more typically three or more separate times, to ensure reproducibility.

DNase I Footprinting, Micrococcal Nuclease Array Disruption Assays, and ChIP

DNase I-primer extension footprinting to examine the binding of TRβ/RXRα to chromatin was performed as described previously (65). Analysis of TRβ/RXRα-induced chromatin remodeling by MNase array disruption assays was performed essentially as described (48, 65). For the ChIP assays, reactions were set up as for the transcription assays except that ribonucleotide 5′-triphosphates were not added. In the experiments with addition of TSA, no acetyl-CoA was added due to the presence of acetyl-CoA in the *Drosophila* S-190. The chromatin was then digested extensively with MNase (10 U/reaction for 10 min at room temperature). The ChIP assays were performed essentially as described (54), except that the DNA in the immunoprecipitated fractions was recovered directly by phenol/chloroform extraction and ethanol precipitation. The immunoprecipitated DNA was then analyzed by slot-blot hybridization with ^{32}P-labeled oligo probes as indicated.

Acknowledgments

We thank Carl Wu and Jim Kadonaga for assistance with the *Drosophila* chromatin assembly extracts, Tory Manning for the p300 proteins, and Mi Young Kim for the SRC-2(RID/PID) and GST-polypeptide inhibitor constructs.

Received September 4, 2002. Accepted February 3, 2003.

Address all correspondence and requests for reprints to: W. Lee Kraus, Department of Molecular Biology and Genetics, Cornell University, 465 Biotechnology Building, Ithaca, New York 14853. E-mail: wlk5@cornell.edu; or Jiemin Wong, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. E-mail: jwong@bcm.tmc.edu.

This work was supported by NIH postdoctoral fellowship F32-DK-59702 (to K.C.L.), NIH Grants R01-DK-56324 and R01-DK-58679 (to J.W.), and NIH Grant R01-DK-58110 and a Career Award in the Biomedical Sciences from the Burroughs Wellcome Fund (to W.L.K.).

* K.C.L. and J.L. are equal contributors.

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